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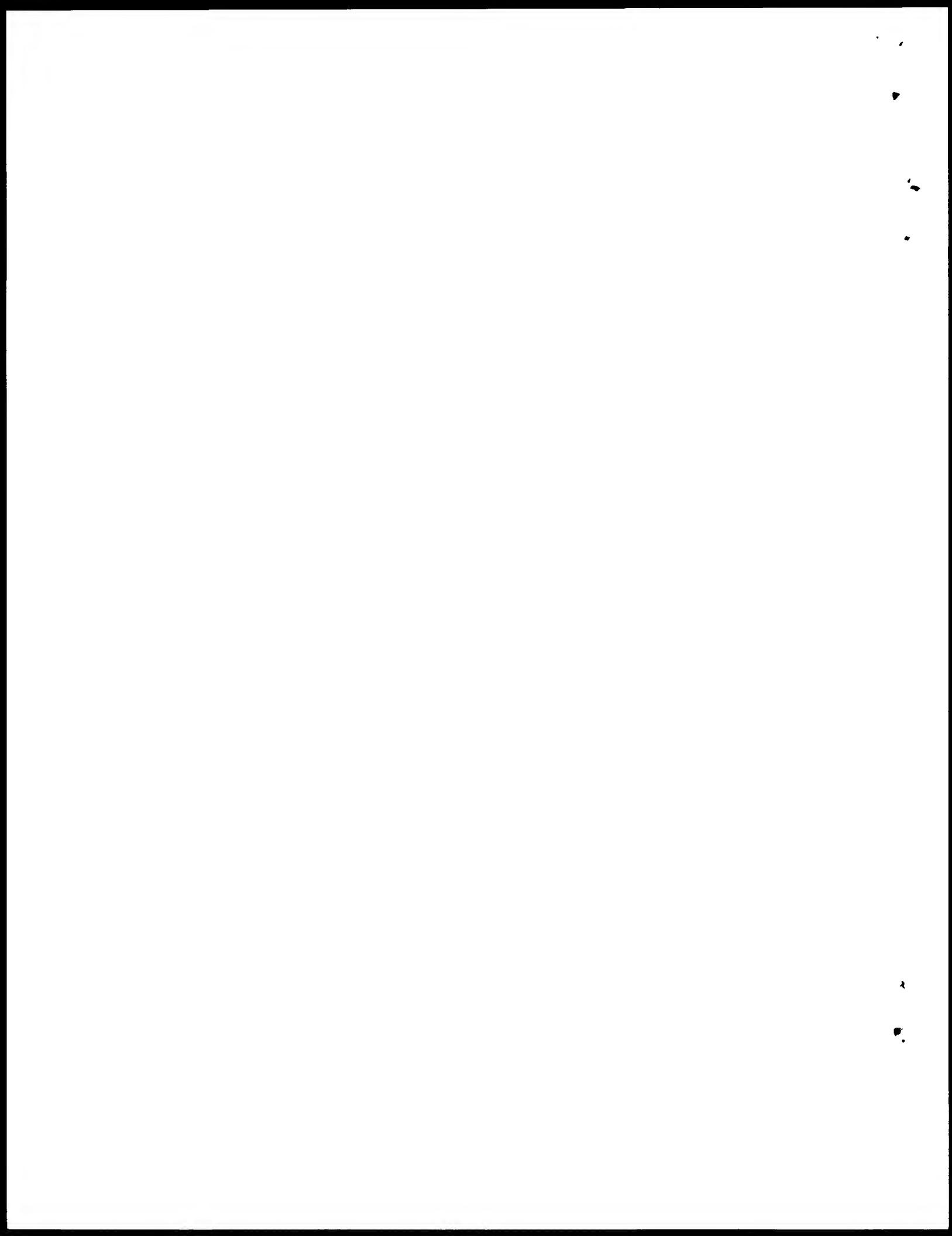
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(54) Title: IMPROVED KERATINOCYTE CULTURE AND USES THEREOF

(57) Abstract: The present invention relates to the treatment of skin defects by organotypically-cultured autologous keratinocytes isolated from the outer root sheath of anagen or growing hair. Methods for primary, as well as subsequent organotypic cultures (i.e., epidermal equivalents) in fully-defined media supplemented by autologous human serum and substances isolated from blood components, with minimal allogeneic biological supplements, are disclosed herein. Techniques to prepare epidermal equivalents for transplantation by use of a biocompatible glue are also disclosed herein.



## IMPROVED KERATINOCYTE CULTURE AND USES THEREOF

### FIELD OF THE INVENTION

The invention relates to the field of cell culture of human keratinocyte precursor and dermal fibroblast cells. The invention also relates to the use of cultured keratinocyte precursor cells in the repair of skin defects by skin grafting procedures.

### BACKGROUND OF THE INVENTION

The healing of skin defects progresses through three general phases: (i) inflammation, (ii) wound cell migration and mitosis, and (iii) extracellular matrix production and remodeling. The ordered sequence of these events is thought to be orchestrated by interactions among cells, growth factors, and extracellular matrix proteins. A crucial step of skin wound healing is epidermal regeneration (*i.e.*, re-epithelialization). Besides interfollicular epidermal keratinocytes from the wound edges, the outer root sheath (ORS) cells from residual hair follicles also contribute to this process (*see e.g.*, Eisen *et al.*, 15 J. Invest. Dermatol. 145-155 (1955)). The ORS of hair follicles is comprised largely of undifferentiated keratinocytes that encompass the cylindrical structures of the hardened inner root sheath and the hair shaft (*see e.g.*, Montagna & Parakkal, In: *The Structure and Function of Skin* 172-258 (Academic Press New York, NY, 1974)). Recent literature has also indicated that ORS cells are at a lower level of commitment to differentiation than the basal interfollicular keratinocytes (*see e.g.*, Coulombe *et al.*, 109 J. Cell Biol. 2295-2312 (1989); Limat *et al.*, 194 Exp. Cell Res. 218-227 (1991); Limat *et al.*, 275 Cell Tissue Res. 169-176 (1994)), and label-retaining cells have been detected in the animal as well as the human ORS region near the bulge area which possibly represent stem cells for skin epithelial tissues (*see e.g.*, Cotsarelis *et al.*, 61 Cell 1329-1337 (1990); Kobayashi *et al.*, 90 Proc. Nat. Acad. Sci. USA 7391-7395 (1993); Yang *et al.*, 105 J. Invest. Dermatol. 14-21 (1993); Rochat *et al.*, 76 Cell 1073-1076 (1994); Moll, 105 J. Invest. Dermatol. 14-21 (1995)). Additionally, human ORS cells which are isolated from plucked anagen scalp hair follicles can be expanded extensively *in vitro* (*see e.g.*, Weterings *et al.*, 104 Brit. J. Dermatol. 1-5 (1981); Limat & Noser, 87 J. Invest. Dermatol. 485-488 (1986); Imcke *et al.*, 17 J. Am. Acad. Dermatol. 779-786 (1987);

Limat *et al.*, 92 J. Invest. Dermatol. 758-762 (1989)). Under conventional submerged culture conditions, ORS cells resemble interfollicular epidermal keratinocytes by both morphologic and biochemical (e.g., keratin profiles) criteria (see e.g., Stark *et al.*, 35 Differentiation 236-248 (1987); Limat *et al.*, 92 J. Invest. Dermatol. 758-762 (1989); Limat *et al.*, 642 Ann. N.Y. Acad. Sci. 125-147 (1991)). In organotypic co-cultures with human dermal fibroblasts (*i.e.*, under conditions mimicking the epidermal environment), ORS cells with respect to histological, immunohistological, ultrastructural and biochemical criteria develop a stratified epithelium reminiscent of regenerating epidermis (see e.g., Lenoir *et al.*, 130 Dev. Biol. 610-620 (1988); Limat *et al.*, 194 Exp. Cell Res. 218-227 (1991); Limat *et al.*, 642 Ann. N.Y. Acad. Sci. 125-147 (1991)). If such organotypic cultures are grafted onto nude mice, ORS cells form a regular neo-epidermis that is under homeostatic control (see e.g., Limat *et al.*, 59 Transplantation 1032-1038 (1995)). Thus, human ORS cells are of considerable interest for clinical application.

In the previous decade, interest has focused on the use of cultured epidermal cells for wound coverage. First, sheets of cultured autologous interfollicular keratinocytes were grafted successfully on acute wounds, mainly in the treatment of larger third degree burns (see e.g., O'Connor *et al.*, 1 Lancet 75-78 (1981); Compton *et al.*, 60 Lab. Invest. 600-612 (1989)) but also of epidermolysis bullosa (see e.g., Carter *et al.*, 17 J. Am. Acad. Dermatol. 246-250 (1987)), pyoderma gangrenosum (see e.g., Dean *et al.*, 26 Ann. Plast. Surg. 194-195 (1991); Limova & Mauro, 20 J. Dermatol. Surg. Oncol. 833-836 (1994)), and wounds after excision of giant congenital nevi (see e.g., Gallico *et al.*, 84 J. Plast. Reconstr. Surg. 1-9 (1989)) or separation of conjoined twins (see e.g., Higgins *et al.*, 87 J. Royal Soc. Med. 108-109 (1994)).

In contrast to the treatment of such acute wounds, the grafting of chronic wounds (e.g., leg ulcers) with cultured keratinocytes has been much less successful. Allografts do not result in a permanent "take" (see e.g., Fabre, 29 Immunol. Lett. 161-166 (1991)) and thus may be classified as a "quite effective but expensive biological dressing" (see Phillips *et al.*, 21 J. Am. Acad. Dermatol. 191-199 (1989). A reproducible, major definite "take" of autologous keratinocyte grafted by various modalities including: sheets of submerged keratinocyte cultures consisting of only a

few, noncornified cell layers (Hetton *et al.*, 14 J. Am. Acad. Dermatol. 399-405 (1986); Leigh & Purkis, 11 Clin. Exp. Dermatol. 650-652 (1986); Leigh *et al.*, 117 Brit. J. Dermatol. 591-597 (1987); Harris *et al.*, 18 Clin. Exp. Dermatol. 417-420 (1993)), trypsinized single cells attached to collagen-coated dressings (Brysk *et al.*, 25 J. Am. Acad. Dermatol. 238-244 (1991)), skin equivalents (Mol *et al.*, 24 J. Am. Acad. Dermatol. 77-82 (1991)) has yet to be convincingly documented within the scientific literature. The same lack of quantitative findings also holds true for various reports on the grafting of freshly isolated, autologous interfollicular keratinocytes (Hunyadi *et al.*, 14 J. Dermatol. Surg. Oncol. 75-78 (1988)) or ORS cells (Moll *et al.*, 46 Hautarzt 548-552 (1995)) fixed to the wound bed by the use of a fibrin glue. However, it should be noted that the disadvantages of the bovine serum used during cultivation of the keratinocytes may contribute to reduced "take" rate, due to the fact that it resists in keratinocytes (*see e.g.*, Johnson *et al.*, 11 J. Burn Care Rehab. 504-509 (1990)).

15

#### SUMMARY OF THE INVENTION

Prior to the disclosure of the present invention herein, the standard methodology for the generation of a primary culture of ORS keratinocytes consisted of the plucking of an anagen (*i.e.*, growing hair shaft) hair followed by a careful microscopic dissection to remove the hair bulbs and the infundibular hair shaft. The resulting outer root sheath was then placed on the culture insert for initiation of the primary keratinocyte culture. However, numerous subsequent studies (approximately 200), wherein the anagen hair was placed directly on the culture insert without performing the initial micro-dissection to remove the hair bulbs and the infundibular hair shaft, have demonstrated that such tedious and time-consuming dissection of the plucked anagen hair was not required. This has served to markedly simplify the handling process, reduce the risk for contamination, and resulted in more efficient initiation of keratinocyte cell plating.

Accordingly, it is an object of the present invention to provide improved and simplified methods for the generation of keratinocytes or keratinocyte precursors from outer root sheath cells (ORS cells) in fully defined culture conditions for the treatment of various types of skin defects (*e.g.*, chronic wounds such as leg ulcers, diabetic

ulcers, pressure sores, and the like) in both humans and animals. In addition to their use in the treatment of wounds, keratinocytes may also be used in plastic and cosmetic surgery, or whenever there is a demand for such skin support (e.g., post operative following the removal of tattoos, naevi, skin cancer, papillomas, after 5 amputation, in sex transformation or re-virgination, rejuvenation of actinically damaged skin after skin resurfacing, tympanoplasty, epithelialization of external ear canal, and the like).

These aforementioned objectives are accomplished by explantation and culture of plucked, anagen or growing hairs *in toto* upon microporous membranes carrying 10 human fibroblast feeder cells at their under-surface. In such primary cultures, large numbers of ORS cells can be easily and repeatedly obtained, irrespective of the donor's chronological age. Such ORS cells may be used for the subsequent preparation of complex skin, *i.e.*, dermo-epidermal, or epidermal equivalents or kept frozen and stored in order to use them at a later time point.

The subsequent preparation of skin or epidermal equivalents is achieved by 15 the "seeding" of these ORS cells upon a modified, microporous membrane carrying fibroblast feeder cells (most preferably growth-arrested/limited human dermal fibroblast "feeder cells") at their under-surface. During culture, these ORS cells undergo tissue differentiation which has been demonstrated to be similar to that of 20 normal epidermis. This finding is most probably due to a large compartment of proliferating cells. The modified culture conditions which are disclosed herein are important for the successful treatment of chronic wounds with epidermal equivalents generated *in vitro* from autologous ORS cells.

A further object of the present invention is to provide improved culture 25 systems for ORS-derived keratinocytes by adhering the anagenic hair onto a polymeric microporous membrane coated with one or more molecules of extracellular matrix origin. These improved cultures of ORS cells, designated as skin equivalents or epidermal equivalents, may be used to treat skin defects, especially chronic wounds.

Yet another object of the present invention is to produce skin or epidermal 30 equivalents using a reduced concentration of allogenic or homologous serum. This greatly mitigates the risk of disease transmission, for example, by clinical use of

blood products, by the use of autologous or homologous human serum and substances derived or released from blood components (e.g., blood platelets) for supplements in *in vitro* culturing steps.

5 A further object of the present invention is a methodology which reduces the probability of mechanical damage (e.g., separation of the various constituent layers) of the skin or epidermal equivalents during transport prior to transplantation.

10 The clinical advantages of the methodology of the present invention, as compared to grafting techniques of chronic wounds which have been previously utilized, include, but are not limited to: noninvasiveness (so that the cells are available repeatedly), the lack of need for surgical facilities or anesthesia during the grafting procedure, and a short immobilization period of only 2 hours required following the grafting procedure.

#### DETAILED DESCRIPTION OF THE INVENTION

15 Unless defined otherwise, all technical and scientific terms used herein have the same meanings commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice of the present invention, the preferred methods and materials are now described. All publications mentioned herein  
20 are incorporated herein in their entirety by reference.

The term "keratinocyte layer" as used herein means an *in vitro* generated keratinocyte tissue culture with more or less differentiated structure. The term "epidermal equivalent" as used herein means an *in vitro* generated organotypic tissue culture resembling in its histological structure the natural epidermis especially concerning the stratification and development of the horny layer. A normal stratified epidermis consists of a basal layer of small cuboidal cells, several spinous layers of progressively flattened cells, a prominent granular layer and an orthokeratotic horny layer. All these layers can be detected in the epidermal equivalents that are subject of the invention. Localization of those epidermal differentiation products that have been assayed by immunohistochemistry (e.g. keratins, involucrin, filaggrin, integrins) is similar to that found in normal epidermis.

The term "autologous" as used herein means: (i) that biological material to be transplanted is derived from the individual to be treated with epidermal equivalents; or (ii) that biological material added to tissue cultures comes from the donor of the cells for tissue culture.

5 The term "homologous" as used herein means: (i) that biological material to be transplanted is derived from one or more individuals of the same species as the individual to be treated with epidermal equivalents; or (ii) that biological material added to tissue cultures comes from one or more individuals of the same species as the donor of cells for the tissue culture.

10 The term "organotypic culture" and the like, refers to culture of cells under conditions that promote differentiation of the cells. Under conditions of organotypic culture, proliferation of the cells is slowed compared to culture under "proliferative" conditions such as primary culture conditions, and may be completely stopped. In the present case, an important condition for organotypic culture is maintenance of the 15 cells at the air-liquid interface, a so-called "lifted" culturing condition.

15 The term "releasate from blood components" (e.g., blood platelets) as used herein means any combination of cytokines or other growth factors obtained from blood components (e.g., blood platelets). Platelets stimulated with, for example, thrombin release the content of their alpha granules into the surrounding medium. 20 Alpha granules usually contain several cytokines (e.g., platelet derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factors alpha and beta (TGF alpha/beta), platelet factor 4 (PF-4), platelet basic protein (PBP)). However, it is possible to obtain cytokines and other growth factors from platelets by other methods than stimulating with thrombin. Moreover, other blood components produce growth 25 factors and cytokines as well. Monocytes, for example, produce IL-1, TNF alpha, IL-6 and other substances of interest.

*General Method for Preparing Epidermal Equivalents from ORS Cells.*

Keratinocyte precursor cells are selected from outer root sheath (ORS) of anagen or growing hair which is derived from the individual which is to be subsequently treated 30 with epidermal equivalents. In general, approximately 40 hair follicles are plucked from the scalp, and those in the anagen phase (*i.e.*, a growing hair shaft) are then selected under the dissecting microscope. A total of four weeks of culture is usually

required in order to obtain approximately 1 cm<sup>2</sup> of epidermal equivalents from five hair follicles. However, with improved culture and fermentation techniques it may be possible to get a higher yield (*i.e.*, a larger area of epidermal equivalents, within this period of time).

5       The previous standard method for the generation of a primary culture of ORS keratinocytes consisted of the plucking of an anagen (*i.e.*, growing hair shaft) hair followed by a careful microscopic dissection to remove the hair bulbs and the infundibular hair shaft. The resulting outer root sheath (ORS) was then placed on the culture insert for initiation of the primary keratinocyte culture. However, numerous 10      subsequent studies (approximately 200), wherein the anagen hair was placed directly on the culture insert without performing the initial micro-dissection to remove the hair bulbs and the infundibular hair shaft, have demonstrated that such tedious and time-consuming dissection of the plucked anagen hair was not required. This has served to markedly simplify the handling process, reduce the risk for contamination, and 15      resulted in more efficient initiation of keratinocyte cell plating.

The selected anagen hairs were incubated in an appropriate rinsing buffer containing various anti-microbial and anti-fungal agents (*e.g.*, fungizone, penicillin, and streptomycin). Following this procedure, the entire plucked anagen hair is placed directly on the culture insert and allowed to grow for several days, preferably 7-14, 20      days, and more preferably 8 to 10 days. An optional, additional step is comprised of passaging the primary culture and performing a secondary culture in order to obtain more cellular material for the preparation of larger areas of epidermal equivalents.

25       The culture insert, a microporous membrane coated with one or more extracellular matrix substances (*e.g.*, fibrin, fibronectin, collagens, laminins or hyaluronan or mixtures thereof), carries a growth-arrested/limited feeder cell system on its undersurface. The coating of the membrane insert with such extracellular matrix substances provides for: (*i*) an enhanced culture surface for the initial attachment of the anagen hair (*i.e.*, it sticks easily and remains stationary); (*ii*) a surface which significantly enhances the migration of the ORS keratinocytes away from the outer root sheath (ORS) anagen hair follicles; and (*iii*) increased growth rates of the spreading ORS keratinocytes (*i.e.*, the overall culture time needed for production of 30

fully differentiated skin or epidermal equivalents) can be reduced to three weeks, instead of four.

The aforementioned growth-arrested/limited feeder cell system located on the under surface of the microporous insert membrane is comprised of primary dermal fibroblasts obtained from a human skin biopsy. The primary dermal fibroblasts are treated with mitomycin-C for 4 to 6 hours prior to their use as a "feeder cell layer" for the plucked anagen hair and then plated on the underside of the culture insert. Growth arrest/limitation is induced by either mitomycin-C or X-ray treatment or, preferably, the reduced serum concentration below 5%, and preferably 2%. It should be noted that, although some cultures had been performed using 10% fetal calf serum (FCS; Boehringer Mannheim, Germany), the current utilization of human serum, in order to reduce the number of allogeneic ingredients, was found to provide markedly superior outgrowth and proliferation of the ORS cells. Moreover, the human serum is preferably utilized in a concentration of less than 5%, and more preferably in a concentration of 2%. In the presence of such low serum concentrations, the primary human dermal fibroblasts of the present invention will become significantly, or completely growth arrested. Hence, in this manner, two expensive and potentially complicating steps in the autologous ORS culture system may be removed. The two complicating steps include: (i) removal of high serum >5% concentrations, which reduces the overall cost of the process significantly and; (ii) the removal of mitomycin-C treatment, which provides a fully mitomycin-C-free culture system and eliminates any concerns regarding the total elimination of the drug from the primary culture inserts prior to the growth of the epidermal equivalents. In addition, the use of reduced serum concentrations allows the alternative feeder cell-arresting procedure (*i.e.*, the X-ray exposure step) to be eliminated, thus saving significant time and expense in the overall procedure.

Following expansion of the ORS cells to an appropriate density (*i.e.*,  $1 \times 10^3$  to  $1 \times 10^6$  cells/cm<sup>2</sup>, and preferably  $5 \times 10^4$  to  $1 \times 10^5$  cells/cm<sup>2</sup>), they are used for preparation of epidermal equivalents. Preferably, the cells are grown to confluence. The epidermal equivalents are prepared by seeding ORS cells at an appropriate cell density (*i.e.*,  $30 \times 10^3$  to  $100 \times 10^3$  cells/cm<sup>2</sup>, and preferably  $60 \times 10^3$  cells/cm<sup>2</sup>) within a culture device which is suitable for "lifting" the cells up to the air-liquid interface

during culture. Subsequently, one to four days after seeding (preferably 3 days after seeding), the ORS cells are exposed to air (e.g., by aspiration of the medium inside the insert) and the cultures are then continued for approximately 10-20 days, and preferably for 14-18 days, in such "lifted" culture condition. The medium is changed periodically during the lifted culture; preferably every two to three days.

The present invention also encompasses skin equivalents which include additional layers, and so are more complex structures than epidermal equivalents. Skin equivalents comprise differentiated ORS cells as their epidermal part and also a layer comprising a matrix component, preferably one containing embedded dermal fibroblasts and/or other cells (*i.e.*, an "embedding matrix"). Skin equivalents are made by placing a matrix with one or more extracellular matrix substances (e.g., fibrin, fibronectin, collagens, laminins or hyaluronan or mixtures thereof) on the upper surface of the microporous membrane described above. When embedding human dermal fibroblasts, preferably autologous human dermal fibroblasts, the cells are embedded at a density of  $1 \times 10^3$  to  $1 \times 10^7$  cells/cm<sup>3</sup>; preferably  $1 \times 10^4$  to  $1 \times 10^5$  cells/cm<sup>3</sup>; and most preferably approximately  $5 \times 10^4$  cells/cm<sup>3</sup>. The primary culture of ORS cells is then seeded on top of the matrix (preferably containing embedded dermal fibroblasts and/or other cells) and organotypic culturing is performed as described above. For a detailed description of the preparation of dermal equivalents (see e.g., Limat *et al.*, 194 Exp. Cell Res. 218-277 (1991)).

It should be noted, however, that the cells which are embedded in the matrix need not be limited exclusively to dermal fibroblasts; as epidermal, mesenchymal, neuronal and/or endothelial cells can also be utilized. The embedded cells are preferably obtained from skin tissue, are more preferably allogeneic cells, and are most preferably autologous cells.

All culture steps are performed in an appropriate medium which allows the proliferation of the ORS cells and their outgrowth from the hair follicles, the medium is typically changed every 2 to 3 days. Generally, the medium utilized for all steps is the same. The medium is typically based on a minimal medium and contains several additional ingredients. One common ingredient is serum in a concentration of 0.5-60%. In the preferred embodiment of the present invention, human serum is used at a concentration of less than 5%, and most preferably at a concentration of 2%.

Furthermore, with the development of serum-free media, it may be possible to omit serum *in toto*. Epidermal growth factor (EGF) stimulates migration of keratinocytes and delays their senescence which results in stimulation of proliferation. Cholera toxin, hydrocortisone, insulin, adenine and triiodothyronine have an effect of stimulating proliferation. All of these ingredients are thus useful in a medium for preparing epidermal equivalents. Nevertheless, it may be possible to omit or replace one or another of these ingredients.

Releasate from blood components (*e.g.*, blood platelets, monocytes or lymphocytes), may serve as a source of cell proliferating activities, and therefore may substitute serum and provide other above mentioned ingredients. For certain culture periods the serum-containing medium might possibly be replaced by a defined, serum-free medium, for example, SFM (Gibco Europe, Ettlingen). The releasate from blood components (*e.g.*, blood platelets, monocytes or lymphocytes), especially of homologous or autologous origin, may serve as a source of cell proliferating activities and therefore may substitute serum and provide other above mentioned ingredients or indeed may provide additional ingredients. The blood components should be added to the culture medium in a concentration of 0.1% to 20%, and preferably 1% to 5%, after the releasate is brought-to the same final volume as the blood from which these components are obtained. These releasates contain several growth factors that are present in serum (*e.g.*, PDGF, ECF or TGFs). However, serum as well as releasates contain many substances, and not all are characterized.

Releasate from blood platelets is obtained by centrifugation of anti-coagulated whole blood, preferably human blood, in order to pellet all cells except thrombocytes. The supernatant is centrifuged once more to spin down the thrombocytes. The thrombocytes are suspended in an appropriate buffer, *e.g.* phosphate buffer and treated with thrombin in order to release their alpha granules which contain a mixture of various growth factors (*e.g.*, PDGF, PF-4, TGF- $\beta$ , EGE,  $\beta$ -thromboglobulin). In a further centrifugation step all cellular material is removed. Finally, the supernatant is supplemented with buffer to the volume of the original blood sample from which the components are obtained. The blood components should be added to the culture

medium in a concentration of 0.1% to 20%; preferably 1% to 10%; and more preferably 2 to 5%.

5           Similarly, releasates can be obtained from other blood cells, such as monocytes, by breaking up the cells (*e.g.*, by sonication, freeze-thaw method, or the like) and purifying the growth factors (*e.g.*, by filtration or immunological methods).

10          The blood component releasates can also be used to condition the wound bed in the course of grafting the epidermal or dermal equivalents. Furthermore, the culture medium containing the releasates and used to perform the organotypic culturing step, after having been conditioned by the cells, can be used to condition the bed of the skin defect in the course of grafting the epidermal or dermal equivalents.

15          Cultivation usually is performed in inserts with microporous membranes, which contain homologous or autologous human dermal fibroblasts (HDF), especially postmitotic HDF at their undersurface. HDF secrete factors that condition the medium in order to get a better growth of the epidermal equivalents. The HDF layer can be formed from between  $5 \times 10^3$  to  $1 \times 10^5$  cells/cm<sup>2</sup>, and preferably approximately  $1 \times 10^4$  to  $5 \times 10^4$  cells/cm<sup>2</sup>. The HDF are preferably postmitotic, but earlier passage cells can be used if they are irradiated, treated with mitomycin-C, or otherwise treated to inhibit their proliferation but maintain their metabolism, *i.e.*, by reduction of serum concentration.

20          In one embodiment, the graft thickness for the complex dermal ("complex skin") equivalents does not exceed 0.4 mm.

25          Microporous membranes are suitable as a culture substrate, because they allow substances to diffuse from one side to the other, but work as a barrier for cells. The pore size of the membrane is not a limitation on the present invention, but should be adequate so as to allow diffusion of proteins of up to 100,000 Daltons molecular weight, and preferably of up to 70,000 Daltons molecular weight. The membrane should at least allow diffusion of small hormones such as insulin, and allow passage of proteins of up to 15,000 Daltons molecular weight. Other means than a microporous membrane for performing the function of allowing diffusion of soluble factors to the cultured ORS cells, while preventing mixing of the ORS cells with the HDF would also be usable.

The microporous membranes typical in the art are usually used. However, membranes fabricated from a biodegradable material (e.g., polyhyaluronic acid or polylactic acid) can also be used. When a biodegradable microporous membrane is employed it is contemplated that the entire culture, including the differentiated ORS cells, the microporous membrane and the HDF, will be transplanted into the skin defect. Thus, in this alternative embodiment, the HDF grown on the underside of the membrane need not be post-mitotic or treated to preclude proliferation. While HDF tend to be less immunogenic than keratinocytes, it is preferable that when this embodiment is employed, the HDF be allogeneic cells, preferably autologous cells.

In one embodiment, the thickness of mesh graft can range from 30-300 microns. Preferably, the mesh graft thickness ranges from 0.5-0.75 mm. A graft of tissue (for example, dermal collagen plus fibroblasts overlaid with keratinocytes tissue) that is too thick can result in a too rapid ischemic cell death, especially for the keratinocyte layer residing above the dermal fibroblast collagen layer. By contrast, this mesh graft tissue can "take" in wound sites.

The epidermal equivalents of the present invention may range in size from approximately 6 mm to approximately 2.5 cm in diameter, with a preferred diameter of 2.5 cm. For practical reasons, the experiments disclosed herein were performed with epidermal equivalents of approximately 2.5 cm in diameter.

In one embodiment, the preferred range for epidermal equivalents is 50-150 microns. In a particular embodiment, the epidermal equivalents are very thin (thinner than is generally used in the art, for example, 60 microns). It has been hypothesized that making the autologous graft too thick will prevent a proper blood supply from being established, so that the epidermis will not "take" at the wound site. By contrast, the epidermal equivalents of the invention can "take" in wound sites.

In many cases, however, the skin or epidermal equivalents will have to be delivered from the facility where they are generated to the institution where they are used. Therefore a system is needed to enable the transport of the skin or epidermal equivalents, which have been kept in a condition ready for grafting. Irrespective of whether the microporous membrane is removed from the basal cell layer before transport, conditions resembling those during cultivation seem to be favorable. In order to keep the skin or epidermal equivalents in contact with medium only from the

basal layer, (*i.e.*, during cultivation), agarose in a concentration ranging from 0.1% to 5%, and preferably in a concentration of 0.5% to 1%, or methyl cellulose, or any other gelling substance in comparable concentrations, may be used to solidify the transport medium. The skin or epidermal equivalents will be placed with their basal layer down on the membrane of an insert previously embedded on top of the solidified or gelled medium. The multiwell dish containing these inserts is then put in a blister sealed by a tyvek cover, and shipped. The skin or epidermal equivalents are, most preferably, used for grafting within 24 to 48 hours of initial packaging.

To improve the stability of the epidermal equivalents, the technique of placing a carrier membrane on top, *i.e.*, onto the cornified aspect, of the epidermal equivalents and eventually adhering to it was developed. As an adhesive, fibrin glue is preferred, however, other options, including, but not limited to: extracellular matrix components such as collagen, fibronectin, proteoglycans (*e.g.*, hyaluronic acid, chondroitin sulfate, and the like), or basement membrane zone components (*e.g.*, laminin, Matrigel<sup>TM</sup>, or L-polylysine), or similar tissue glues, may also be utilized.

The carriers utilized in the present invention may consist of a synthetic membrane, made from at one or more of the following materials (polyester, PTFE or polyurethane); from one or more biodegradable polymers (*e.g.*, hyaluronic acid, polylactic acid or collagen); or a silicone or vaseline gauze dressing, or any other material suitable for wound dressing. These materials which are suitable for wound dressing allow the carrier to remain in place to immobilize the implanted dermal or epidermal equivalents for several days, rather than requiring the carrier to be removed immediately after the dermal or epidermal equivalents are transplanted. Thus, the carrier not only enhances stability and improves handling, but it also serves as a protective coat against physical damage as well as the proteolytic milieu and bacteria in the wound. Moreover, it serves for orientation of the graft (*i.e.*, basal side down, cornified side up).

The skin or epidermal equivalents put onto the carrier have to be kept in a condition ready for grafting. Irrespective of whether the microporous membrane is removed from the basal cell layer for transport, conditions resembling those during cultivation seem to be favorable. In order to keep the skin or epidermal equivalents in contact with medium only from the basal layer (*i.e.*, during cultivation), agarose in a

concentration ranging from 1% to 5%, and preferably in a concentration of 1 to 3%; methyl cellulose; or any other gelling substance in comparable concentrations, may be used to solidify the medium. The epidermal equivalents together with the carrier will be placed with their basal layer on top of the solidified or gelled medium. The whole device is then sealed in an air tight manner, and shipped. The epidermal equivalents are, most preferably, used for grafting within 24 hours of initial packaging.

The skin or epidermal equivalents are transplanted by simply placing them in the bed of the wound or other skin defect. Preferably the skin or epidermal equivalents are then immobilized (patients are immobilized for 2 hours). The preferred method for immobilization is by use of a biodegradable material, by some sort of tissue glue or adequate bandage. As previously described, the bed of the skin defect can be treated with blood releasates or the medium from the organotypic culturing prior to, or concomitantly with, the transplantation.

In work using encapsulated cells devices (100 micron membrane, 200-250 microns to the center of the hollow fiber), good survival of human dermal fibroblasts has been obtained at 300 micron distances from the nearest blood vessel.

#### EXAMPLE 1 PREPARATION OF ORS CELLS

Keratinocyte precursor cells from the outer root sheath (ORS) of the hair follicles are selected and subsequently cultured by use of the following methodology, as disclosed in the present invention.

Approximately 40 hair follicles were plucked with tweezers from the occipital scalp of individuals, and those in the anagen phase, as detected, for example, by well-developed root sheaths, were then selected under the dissecting microscope (see e.g., Limat & Noser, 87 J. Invest. Dermatol. 485-488 (1986); Limat *et al.*, 92 J. Invest. Dermatol. 758-762 (1989)). The anagen hair was placed directly on the microporous culture insert without performing the previously-utilized micro-dissection to remove the hair bulbs and the infundibular hair shaft.

Generally, six anagenic hairs were explanted on the microporous membrane of a cell culture insert (Costar) that carried on its undersurface a preformed feeder layer

preferably comprised of  $20 \times 10^3$  postmitotic human dermal fibroblasts (HDF) per cm<sup>2</sup>. (see e.g., Limat *et al.*, 92 J. Invest. Dermatol. 758-762 (1989)). The HDFs were derived from skin explants of a healthy, repeatedly HIV- serology negative and hepatitis-serology negative individuals and cultured in DMEM supplemented with 10% fetal calf serum (FCS), or preferably less than 5% human serum, or most preferably 2% human serum.

For the purpose of obtaining an efficient outgrowth of the outer root sheath (ORS) cells from the anagen hair and a high proliferation rate, it is important not to place the HDF feeder cells at the bottom of the culture dish, resulting in an additional medium layer between the HDF layer and the microporous membrane supporting the ORS cells. Growing each cell type at one side of the microporous membrane allows a very close interaction, but prevents cross contamination of the ORS cells with fibroblasts and thus guarantees a pure culture of ORS cells.

The culture medium which was utilized consisted of Dulbecco's modified Eagle's medium/F12 (3:1 v/v) supplemented with 2% human serum, 10 ng of epidermal growth factor per ml of culture medium, 0.4 microgram of hydrocortisone per ml, 0.135 mM adenine, and 2 nM triiodothyronine (all obtained from Sigma Chemical Co., St. Louis, MO). The preferred final Ca<sup>2+</sup> concentration of the culture medium is 1.5 mM (see e.g., Wu *et al.*, 31 Cell 693-703 (1982); Limat & Noser, 87 J. Invest Dermatol. 485-488 (1986)). Within about 2 weeks, the ORS cells had expanded and reached confluence. They were dissociated with 0.1% trypsin/0.02% EDTA mixture, checked for viability, and used for preparation of epidermal equivalents. It should be noted that, although initial cultures had been performed using 10% fetal calf serum (FCS; Boehringer Mannheim, Germany), current utilization of human serum, in order to reduce the number of allogeneic ingredients, provided superior outgrowth and proliferation of the ORS cells. The human serum is preferably utilized in a concentration of less than 5%, and most preferably in a concentration of 2%.

Explanting plucked anagen hairs directly on the membrane of culture inserts carrying postmitotic HDF on the undersurface as feeder cells proved to be a simple, efficient, and reproducible method for establishing primary cultures of ORS cells. Approximately 80% of the explanted hair follicles gave rise to outgrowth of ORS cells, even when derived from individuals aged more than 90 years. After 14 days,

large areas of the insert were covered by compactly arranged small cells, at which time they were used for preparation of epidermal equivalents of the present invention.

The comparison of the growth behavior of 70 strains of ORS cells, which were derived from a total of 30 donors, demonstrated no significant differences between the young (*i.e.*, 21 donors aged 19-50 years) and the old (*i.e.*, 9 donors aged 51-93 years) donors. Approximately  $5 \times 10^5$  cells were generally obtained per explanted follicle and the overall degree of cell viability was typically higher than 95%. In contrast, in the absence of postmitotic HDF as a feeder layer, there was only sporadic outgrowth of ORS cells from the explanted follicles.

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#### EXAMPLE 2 PREPARATION OF EPIDERMAL EQUIVALENTS

ORS cells harvested from primary cultures were seeded at a density of  $30 \times 15 10^3$  cells/cm<sup>2</sup> to  $100 \times 10^3$  cells/cm<sup>2</sup>, and preferably  $60 \times 10^3$  cells/cm<sup>2</sup>, on cell culture inserts (Costar) which had been previously inoculated with  $10 \times 10^3$  cells/cm<sup>2</sup> to  $50 \times 20 10^3$  cells/cm<sup>2</sup>, and preferably  $20 \times 10^3$  cells/cm<sup>2</sup>, of postmitotic HDF cells on the undersurface of their microporous membrane. Similar to the culture of ORS cells, it is important to keep the HDF feeder cells in close proximity with the ORS cells, while concomitantly keeping them separated by use of the microporous membrane. This culture technique enhances proliferation, differentiation, and thus the homeostasis of the developing tissue.

Culture medium was identical that utilized for the preparation of the primary cultures as described *supra*. After 72 hours, the ORS cells were exposed to air by aspiration of the liquid medium inside the insert (*i.e.*, leaving the underside of the insert in contact with medium) and cultured for an additional 12-14 days, with three medium changes per week. Alternatively, after one week lifted culture serum may be totally omitted.

For transplantation, the so-far-utilized protocol, which is generally employed for preparation of the fully differentiated epidermal equivalent for wound grafting, requires the physician to carefully cut the entire perimeter of the culture insert with a scalpel blade so as to facilitate the release of the insert membrane (with undercoated human dermal fibroblasts) with the attached skin patch squamous-side up. The skin

patch is then released from this membrane by peeling with a fine forceps and placed, basal-side up, on a new membrane disk in a culture dish for eventual transplant to the patient. This aforementioned procedure is both laborious and time consuming, and can lead to reversal of the basal and squamous orientation.

5 A markedly simpler method which utilizes a carrier membrane patch cap has been devised which utilizes a membrane patch cap (analogous to the fibrin glue patch procedure described below) which is placed directly on top of the squamous surface layer. The membrane cap can then be easily grasped together with the skin patch below using fine forceps and peeled from the culture insert well surface, and, e.g., after incubation in diluted Dispase solution, be peeled from the culture insert membrane. The membrane can then serve a plate for placing the graft onto the wound without mixing up the orientation of the graft (*i.e.*, basal side down, squamous side up).

10  
15 For stabilization and as a protective coating in case of grafting, the epidermal equivalents of the present invention are coated on top with diluted fibrin glue, which also serves to clearly identify the upper (*i.e.*, cornified) side. Fibrin glue, the preferred embodiment of the present invention, is a generally accepted, natural human product which is used extensively as a tissue glue. By applying a thin coating of fibrin glue (which is clearly visible with the naked eye) to the cornified squamous air-exposed surface of the epidermal equivalent, the physician placing the epidermal equivalent onto the wound site will be fully assured of proper graft orientation (*i.e.*, the basal surface of the skin patch will always be the side that does not have the clearly visible fibrin glue cap). Previously, in many instances, during the preparation of the epidermal patch for wound grafting, the orientation of the patch becomes confused.  
20  
25 Should the skin patch be placed in squamous-side down orientation onto the graft site, there would be significantly decreased likelihood of a successful graft. Thus, the use of this simple "marking" completely eliminates this problem.

30 In addition, anti-microbial and/or anti-fungal substances may also be included in the fibrin glue, so as to impede any possible microbial contamination or overgrowth of the graft. Many chronic lesions are chronically-infected, which can result in the inhibition of graft "take" and subsequent wound healing following the initial skin grafting. Moreover, the addition of one or more antibiotics or anti-fungal agents by

direct emulsification within the fibrin glue surface cap, may provide a significant improvement in the delivery of sufficient quantities of anti-microbial agents to the transplant site.

It should be noted that the ORS cells which were harvested from primary cultures, and cultured at the air-liquid interface on insert membranes carrying postmitotic HDF at their undersurface, typically developed a stratified epithelium within 14 days. This stratified epithelium consisted of a basal layer of small cuboidal cells below a thick suprabasal compartment of progressively flattened cells. A prominent granular layer, as well as an orthokeratotic horny layer were also found to be present.

Based upon the experimental finding of approximately 80% of the follicles giving rise to ORS cell outgrowth, approximately five anagen hair follicles were required for the generation of 1 cm<sup>2</sup> of epidermal equivalents. The period to generate graftable epidermal equivalents usually was four weeks *in toto* (*i.e.*, two weeks for the primary culture and two weeks for the subsequent organotypic culture).

### EXAMPLE 3 STABILIZATION

Before delivery, the epidermal equivalents are "coated on-top" by placing a silicone membrane of an appropriate diameter onto the cornified upper aspect of the cultures. To further enhance stability, *e.g.*, in case of thin and/or large epidermal equivalents, as well as to increase adhesion of the silicone membrane, a thin layer of tissue glue, *e.g.* fibrin glue, may be applied before.

On-top coating (1) enhances stability and improves handling of the grafts, and (2) serves as a protective coat against physical damage as well as the proteolytic milieu and bacteria in the wound.

### EXAMPLE 4 SHIPPING

On-top coated epidermal equivalents are detached from the culture insert membranes by incubation in diluted Dispase and then grasping the epidermal equivalents together with the silicone membrane using fine tweezers and transferring

them on the membrane of an insert previously embedded in 0.7% agarose soaked with culture medium in the well of a multiwell dish. These dishes are then placed in the shipping container. For application to the wound bed, the epidermal equivalents are again grasped, together with the silicone membrane, which (1) serves for orientation of the graft (*i.e.*, basal side down, cornified side up) and (2) by leaving it on the grafted epidermal equivalents in the wound serves as a protective coat (*see* above).

#### EXAMPLE 5

#### SUCCESSFUL TREATMENT OF CHRONIC LEG ULCERS WITH EPIDERMAL EQUIVALENTS GENERATED FROM CULTURED AUTOLOGOUS OUTER ROOT SHEATH CELLS

The outer root sheath cells of hair follicles can substitute for interfollicular epidermal keratinocytes, as during healing of skin wounds when these cells migrate onto the denuded area and contribute to epidermal regeneration (Limat *et al*, 107(1) J. Invest. Dermatol. 128-35 (1996), incorporated by reference). Using the improved culture techniques of the invention, we generated epidermal equivalents from cultured outer root sheath cells of patients suffering from recalcitrant chronic leg ulcers, primarily of vascular origin. In such epidermal equivalents, tissue organization as well as immunolocalization of epidermal differentiation products (keratin 10, involucrin, filaggrin) and integrins were indistinguishable from normal epidermis. As determined by the number of bromodeoxyuridine-incorporating cells, the basal layer contained a large compartment of proliferative cells irrespective of donor age. FACS analysis of the outer root sheath cells, used to prepare the epidermal equivalents, disclosed a fraction of small cells with enhanced expression of  $\beta 1$ -integrin, a potential stem cell marker. In contrast to acute wounds, a major definitive take of grafted cultured autologous keratinocytes has not been convincingly demonstrated in chronic wounds. Grafting of epidermal equivalents generated *in vitro* from autologous outer root sheath cells on 11 ulcers in five patients resulted in a definitive take rate of about 80%, with subsequent complete healing within 2 to 3 weeks of five out of seven ulcers grafted with densely arranged cultures. This improvement in the treatment of chronic leg ulcers with cultured autologous keratinocytes probably depends on the large compartment of proliferative cells as well as on a well-developed horny layer.

which prevents disintegration of the grafts. Practical advantages of the new technique are its noninvasiveness, the lack of need for surgical facilities or anesthesia, and a short immobilization period after grafting.

*In Vitro Experiments. Cell Cultures.* About 40 hair follicles were plucked from the occipital scalp of individuals aged up to 91 years, and those in the anagen phase selected under the dissecting microscope. The hair bulbs as well as the infundibular parts were removed with microsurgical blades. Usually, six follicles were explanted on the microporous membrane of a cell culture insert (Falcon 3090; Becton Dickinson, Franklin Lanes, NJ) that carried on its undersurface a performed feeder layer made of  $10^5$  postmitotic human dermal fibroblasts. Culture medium consisted of Dulbecco's modified Eagle's medium/F12 (3:1) supplemented with 10% fetal calf serum (Boehringer Mannheim, German), 10 ng of epidermal growth factor per ml, 0.4  $\mu$ g of hydrocortisone per ml, 0.1 nM cholera toxin, 0.135 mM adenine, and 2 nM triiodothyronine (all from Sigma Chemical Co., St. Louis, MO), final  $\text{Ca}^{2+}$  concentration 1.5 mM. Within about 2 wk, the ORS cells expanded and reached confluence. They were dissociated with trypsin/EDTA 0.1%/0.02%, checked for viability, and grown either in secondary cultures in keratinocyte growth medium (KGM containing 0.15 mM  $\text{Ca}^{2+}$ ; PromoCell, Heidelberg, Germany) or used for flow cytometry analysis and preparation of epidermal equivalents (see, below). For long-term storage in liquid nitrogen, they were frozen in KGM containing 10% fetal calf serum and 10% dimethylsulfoxide.

For comparison, primary cultures of ORS cells were also established by trypsinization of hair follicles and plating the disaggregated ORS cells on a preformed feeder layer made of postmitotic fibroblasts, as previously described (Limat *et al.*, 1989). To avoid confusion, follicles obtained by this method are referred to as "trypsin-treated follicles."

Fibroblasts were derived from skin explants of a healthy, HIV-serology, and hepatitis-serology-negative individual and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

*Flow Cytometry.* The following mouse monoclonal antibodies (mAbs) of IgG<sub>1</sub> subtype reacting with different integrin chains were used: 4B4 with the  $\beta_1$ -chain

(Coulter, Hialeah, FL), 5E8 with the  $\alpha_2$ -chain, J143 with the  $\alpha_3$ -chain, Lv 230 with the  $\alpha_v$ -chain, and MT78 with the  $\alpha_6$ -chain. MAb 439-9B recognizes the  $\beta_4$ -chain.

ORS cells at  $1 \times 10^6/\text{ml}$  were washed once with phosphate-buffered saline, 1% fetal calf serum, and 0.02%  $\text{NaN}_3$  at  $4^\circ\text{C}$  and reconstituted with 1 ml of the same buffer. A 100  $\mu\text{l}$  cell suspension was then incubated with 0.1  $\mu\text{g}$  of mAbs or isotype control antibody (Dako, Glostrup, Denmark) for 25 min at  $4^\circ\text{C}$ . After being washed twice with the same buffer, cells were incubated with a phycoerythrin-labeled polyclonal goat anti-mouse anti-body (Dako) for another 25 min at  $4^\circ\text{C}$ , washed again, and subsequently fixed with the above-mentioned buffer supplemented with 2% paraformaldehyde. Cells were analyzed on a 4-logarithmic scale EPICS Profile II flow cytometer equipped with a power pack, and data were analyzed using the ELITE software (Coulter).

*Epidermal Equivalents.* ORS cells harvested from primary cultures were seeded at a density of  $5 \times 10^5/\text{cm}^2$  on cell culture inserts (Falcon 3095) carrying  $5 \times 10^4$  postmitotic fibroblasts on the undersurface of their microporous membrane. Culture medium was the same as for the preparation of primary cultures. After 24 hr, the ORS cells were exposed to air by aspiration of the medium inside the insert and then cultured for 12 to 14 days with three medium changes per week. In some cultures, 65  $\mu\text{M}$  5-bromo-2'-deoxyuridine (BrdU; Sigma) were added for the final 18 hr.

For histologic analysis, the epidermal equivalents were excised from the insert with a 6 mm punch (Stiefel Laboratorium, Offenbach am Main, Germany), fixed in 5% formalin, and processed further together with the underlying insert membrane according to standard procedures. For immunohistologic examination, the epidermal equivalents were similarly punched out, but then separated from the insert membrane by fine tweezers, snap-frozen in liquid nitrogen-cooled isopentane, and stored at -80°C until processing.

For indirect immunofluorescence, cryostat sections of 6  $\mu\text{m}$  were air-dried, fixed with ice-cooled acetone/ethanol (1:1), rehydrated with phosphate-buffered saline, blocked for 15 min with nonimmune serum, and incubated at room temperature for 60 min with the primary antibodies and, after extensive washing, for

45 min with the secondary antibodies. The following mAbs were used as primary antibodies: Ks 8.60, mainly reacting with keratin (K) 10 and weakly with K1, diluted 1:20 (Sigma); anti-human involucrin, diluted 1:100 (Sigma); anti-human profilaggrin/filaggrin, diluted 1:100 (BTI, Stoughton, MA); 4B4 directed against the  $\beta_1$ -integrin chain, diluted 1:10 (Coulter). Secondary mAbs against mouse IgG conjugated with fluorescein isothiocyanate were purchased from Sigma. As negative controls, sections were incubated with non-immune serum and conjugated secondary antibodies, which revealed in a few cases weak diffuse staining of fully keratinized areas.

For the determination of BrdU-positive cells, cryostat sections were denatured in 1.5 M HC1 and successively incubated with 0.5  $\mu$ g/ml Hoechst 33258 for 30 min, mAb anti-BrdU (Partec, Arlesheim, Switzerland) diluted 1:100 for 45 min, and fluorescein isothiocyanate-linked anti-mouse IgG (Sigma) diluted 1:30 for 45 min. The percentage of BrdU-positive cells in the basal layer was determined in epidermal equivalents prepared from ORS cells of two leg ulcer patients aged 72 and 91 years ( $n = 4$ ; two epidermal equivalents per patient). For each epidermal equivalent, about 2500 basally located nuclei in 10 randomly selected sections were counted.

For transplantation, the epidermal equivalents were excised from the insert together with the underlying membrane using a 6-mm punch (Stiefel Laboratorium) and positioned upside-down on a punched-out polyester membrane (Thomapor 95877; Reichelt Chemie, Heidelberg, Germany) of 6 mm diameter. In one patient, additional epidermal equivalents of 8 mm diameter were prepared likewise. The insert membrane together with the attached postmitotic fibroblasts was then carefully removed with fine tweezers. The epidermal equivalents on their supporting polyester membrane were washed in Dulbecco's phosphate-buffered saline and left floating therein until their application on the wound bed, usually for no longer than 30 min.

*Autologous Grafting in Chronic Leg Ulcers.* With the approval of the Ethics Committee of the University of Berne and after obtaining written informed consent, five in-patient (one male, four females, aged 58 to 91) suffering from recalcitrant chronic leg ulcers (four of them with more than two ulcers on the same leg, duration at least 4 years; venous or mixed arterial and venous disease in four, in one additional

diabetes mellitus, primary lymphoedema in one) were enrolled in a pilot study. The ulcers were cleaned conventionally (primarily with hydrocolloidal dressings and topical antimicrobial agents) until ready for grafting. Then up to 20 autologous epidermal equivalents, usually 6 mm, in one ulcer 8 mm in width, were placed, basal layer downward on the surface of the ulcers, and the supporting polyester membranes were carefully removed with fine tweezers. This grafting procedure was performed at the bedside; no anesthesia was needed. In four of the patients, further ulcers on the same leg served as controls. All ulcers were then covered with a transparent, semiocclusive dressing (Tegaderm; 3M, London, Canada) overlaid by an elastic bandage with compression adapted to the patient's arterial status. The patients were immobilized for 2 h immediately after grafting. After 3 d, the semiocclusive dressing was carefully removed and a hydropolymer dressing (Tielle: Johnson & Johnson Medical, Ascot, UK) applied, again overlaid by the elastic bandage. The hydropolymer dressings were then changed every 2 to 5 days. After complete re-epithelialization local treatment was switched to topical emollients, and the patients were instructed to adhere to a long-term compression therapy adapted to their arterial status. Take of the grafts and healing of the ulcers was documented by standardized photographs taken on each change of the dressings.

*In Vitro ORS Cells Differentiate Into Epidermal Equivalents Similar to Normal Epidermis.* Explanting plucked anagen hair follicles directly on the membrane of culture inserts carrying postmitotic fibroblasts as feeder cells at their undersurface proved to be a simple, efficient, and reproducible tool for establishing primary cultures of ORS cells. About 80% of the explanted hair follicles gave rise to outgrowth of ORS cells, even when derived from individuals aged up to 91 years. After 14 days, large areas of the insert were covered by compactly arranged small cells, at which time they were used for the preparation of the epidermal equivalents. In contrast, ORS cells derived from the trypsin-treated follicles exhibited a less compact arrangement with numerous cells of a larger size. Comparison of the growth behavior of 70 strains of ORS cells derived from 30 donors revealed no significant differences between young (21 donors aged 19 through 50 years) and old donors (9 donors aged 51 through 93 years), since about  $0.5 \times 10^6$  cells were usually obtained

per explanted follicle. Cell viability was higher than 95%. In the absence of postmitotic fibroblasts, there were only sporadic outgrowth of ORS cells.

Because a logarithmic linear relationship between the relative level of  $\beta_1$ -integrin on the cell surface and the proliferative capacity of keratinocytes has been postulated, we compared the expression of integrins in primary cultures of ORS cells established by the two different techniques, i.e., ORS cells from explanted follicles or from trypsin-treated follicles. ORS cells from four different donors grown by both techniques were analyzed by flow cytometry. On the basis of their light-scattering characteristics, the cells could be subdivided into two groups; group A, with a distinctly lower forward light scatter, *i.e.*, smaller cell size, and group B, with higher forward light scatter, thus having a larger cell size. For ORS cells derived from explanted follicles, group A accounted for about 4% and group B for 72% of the total cell number, while values of 2.6% and 75%, respectively, were found for ORS cells grown from trypsin-treated follicles (mean values of four separate experiments). In group A, the percentage of cells staining for  $\beta_1$ - $\beta_4$ -integrins as well as the mean fluorescence per cell of the  $\beta_1$ - and to a lesser extent also the  $\alpha_2$ -,  $\alpha_3$ -,  $\alpha_v$ -integrins, were higher in ORS cells grown from explanted follicles than in those from trypsin-treated follicles. In group B, no differences were detected in the two culture techniques, neither in the percentage of integrin-positive cells nor in the mean fluorescence per cell.

ORS cells harvested from primary cultures and plated on insert membranes carrying postmitotic fibroblasts at their undersurface developed a stratified epithelium within 14 days. This consisted of a basal layer of small cuboidal cells below a thick suprabasal compartment of progressively flattened cells. A granular layer and a mostly orthokeratotic horny layer were present.

The immunolocalization of epidermal differentiation products was identical to that found in normal epidermis. Thus, the differentiation-specific K10 was absent in the basal layer, but strongly expressed suprabasally from the second layer on. Involucrin displayed its typical honey-comb pattern from the mid-stratum spinosum on, whereas the granular staining of filaggrin formed a continuous band beneath the horny layer. As in normal epidermis, the reactivity of the  $\alpha_2$ -,  $\alpha_3$ - and  $\beta_1$ -chains of

integrins was distributed over all aspects of the plasma membrane of the basal cells, displaying decreasing intensity with progressive differentiation.

5 BrdU-positive cells were found predominantly in the basal layer of the epidermal equivalents and accounted for 24% of the basal cells [597 ± 21 BrdU-positive cells for 2464 ± 115 basal cells (mean ± SD): n = 4].

Based on 80% of follicles giving rise to ORS cell outgrowth, about five anagen hair follicles were needed to generate 1 cm<sup>2</sup> of epidermal equivalents. The period to generate graftable epidermal equivalents usually was 4 weeks *i.e.*, 2 weeks for the primary culture and 2 weeks for the organotypic culture.

10 *Autologous Epidermal Equivalents Are Grafted Successfully on Chronic Leg Ulcers.* A total of 11 ulcers were treated, seven of them by covering about 90% of the ulcer surface with densely arranged cultures, four by putting isolated cultures into the central parts. On the first change of the dressing 3 d after grafting, about 80% of the grafts were visible and adherent to the wound bed in both types of treatment. Within 15 the following 2 to 3 wk the grafts consolidated in five of the seven densely grafted ulcers, resulting in complete re-epithelialization and healing. In the two remaining, chronically infected (*Pseudomonas*) ulcers, the grafts were partly destroyed, which led to delayed healing by 4 to 5 weeks. In the ulcers treated by isolated grafts, there was accelerated formation of granulation tissue and re-epithelialization mainly from 20 the wound edges, as compared to the ulcers on the same leg treated with the dressings only. In this type of treatment, permanent take with subsequent expansion of the grafts resulting in complete re-epithelialization was only documented for one ulcer treated with larger epithelial sheets measuring 8 mm in diameter. The control ulcers in the four patients with more than two ulcers on the same leg were only slightly 25 improved after 3 weeks, at which time they were treated either by further grafting of autologous epidermal equivalents or by conventional surgery.

After re-epithelialization, the epidermis was initially still fragile with some tendency to blistering after minor frictional trauma, occasionally resulting in small erosions. These erosions re-epithelialized rapidly under conventional topical treatment. The first patients have now been followed up for 6 mo and show increasing 30 stabilization of the treated areas and no recurrence of the ulcers so far.

From the foregoing detailed description of the specific embodiments of the present invention, it should be readily apparent that a unique methodology for the selection and culture of keratinocytes from the outer root sheath (ORS) of hair follicles for subsequent use in, for example, skin grafting procedures, has been described. Although particular embodiments have been disclosed herein in detail, this has been done by way of example for purposes of illustration only, and is not intended to be limiting with respect to the scope of the appended claims which follow. In particular, it is contemplated by the inventor that various substitutions, alterations, and modifications may be made to the invention without departing from the spirit and scope of the invention as defined by the claims. For example, the selection of anagen hairs are believed to be a matter of routine for a person of ordinary skill in the art with knowledge of the embodiments described herein.

## CLAIMS

### WE CLAIM:

- 5        1. A method for the treatment of a skin defect comprising the application, to said defect, of a portion of an epidermal or complex skin equivalent comprising keratinocyte precursor cells derived from the culturing of outer root sheath cells which were initially derived from the culturing, *in toto*, of an anagen or growing hair.
- 10        2. The method of claim 1, wherein said outer root sheath cells are autologous cells derived from the individual who will subsequently undergo treatment for a skin defect.
- 15        3. The method of claim 1, wherein said outer root sheath cells are homologous cells.
- 20        4. The method of claim 1, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing only homologous or autologous biological supplements.
- 25        5. The method of claim 1, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing human serum in a concentration of less than 5%.
- 30        6. The method of claim 1, wherein the culture density of said keratinocyte precursor cells is between about  $3 \times 10^4$  cells/cm<sup>2</sup> and about  $1 \times 10^5$  cells/cm<sup>2</sup>.
7. The method of claim 1, wherein said epidermal or skin equivalents are coated on their top or cornified side with a fibrin glue.
8. The method of claim 1, wherein said epidermal or skin equivalents are coated on their top or cornified side with a carrier membrane.

9. The method of claim 1, wherein the graft thickness for said epidermal equivalents is 50-150 microns.
- 5 10. The method of claim 1, wherein the graft thickness for said complex skin equivalents does not exceed 0.4 mm.
- 10 11. A method for the treatment of a skin defect comprising the application, to said defect, of a portion of an epidermal or skin equivalent comprising keratinocyte precursor cells derived from the culturing of outer root sheath cells which were initially derived from the culturing of an anagen or growing hair; wherein all culturing of cells is performed in a medium which utilizes autologous or homologous human serum in a concentration of less than about 5%.
- 15 12. The method of claim 11, wherein said anagen or growing hair is cultured *in toto*.
- 20 13. The method of claim 11, wherein said outer root sheath cells are autologous cells derived from the individual who will subsequently undergo treatment for a skin defect.
- 25 14. The method of claim 11, wherein said outer root sheath cells are homologous cells.
15. The method of claim 11, wherein the culture density of said keratinocyte precursor cells is between about  $3 \times 10^4$  cells/cm<sup>2</sup> and about  $1 \times 10^5$  cells/cm<sup>2</sup>.
- 30 16. The method of claim 11, wherein said epidermal or skin equivalents are coated on their top or cornified side with a fibrin glue.

17. The method of claim 11, wherein said epidermal or skin equivalents are coated on their top or cornified side with a carrier membrane.
- 5 18. The method of claim 11, wherein the graft thickness for said epidermal equivalents is 50-150 microns.
19. The method of claim 11, wherein the graft thickness for said complex skin equivalents does not exceed 0.4 mm.
- 10 20. A method for the treatment of a skin defect comprising the application, to said defect, of a portion of an epidermal or skin equivalent comprising keratinocyte precursor cells derived from the culturing of outer root sheath cells which were initially derived from the culturing of an anagen or growing hair; wherein said epidermal or skin equivalent are coated on their top or cornified side with a fibrin glue.
- 15 21. The method of claim 20, wherein said anagen or growing hair is cultured *in toto*.
- 20 22. The method of claim 20, wherein said outer root sheath cells are autologous cells derived from the individual who will subsequently undergo treatment for a skin defect.
- 25 23. The method of claim 20, wherein said outer root sheath cells are homologous cells.
24. The method of claim 20, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing only homologous or autologous biological supplements.

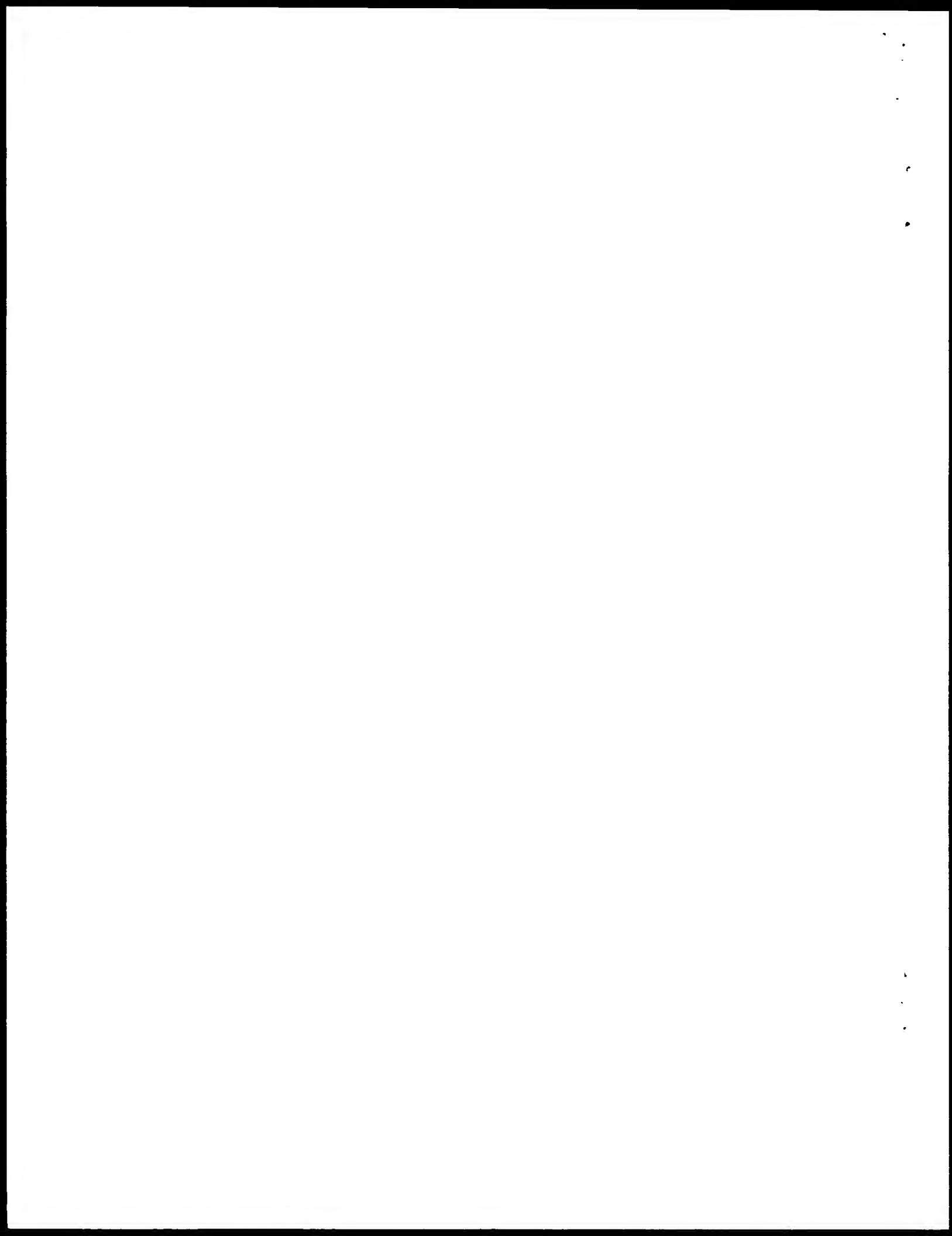
25. The method of claim 20, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing human serum in a concentration of less than 5%.
- 5 26. The method of claim 20, wherein the culture density of said keratinocyte precursor cells is between about  $3 \times 10^4$  cells/cm<sup>2</sup> and about  $1 \times 10^5$  cells/cm<sup>2</sup>.
- 10 27. The method of claim 20, wherein said epidermal or skin equivalents are coated on their top or cornified side with a fibrin glue which contains one or more anti-microbial, anti-fungal, or anti-viral agents emulsified therein.
28. A method for the selection of keratinocyte precursor cells from the outer root sheath of hair for subsequent use in a composition for healing a skin defect, comprising the steps of:
  - 15 (a) plucking of an anagen or growing hair;
  - (b) primary-culturing the outer root sheath-derived keratinocyte precursor cells by adhering said anagen hair, *in toto*, to a microporous membrane, which possesses growth-arrested/limited feeder cells on its undersurface so as to select for keratinocyte precursor cells from the outer root sheath of hair;
  - 20 (c) organotypically-culturing the outer root sheath cells harvested from said primary cultures by inoculating a microporous membrane which also possesses growth-arrested/limited feeder cells on its undersurface;
  - (d) generating an epidermal or complex skin equivalent, for subsequent use as a graft insert, comprised of keratinocyte precursor cells by placing a carrier membrane on top of said organotypic-culture from step (c) and detaching said skin or epidermal equivalent, which is comprised of the keratinocyte precursor cells and carrier membrane, together as a single, laminar unit;
  - 25 (e) contacting said epidermal or skin equivalent with a skin defect present on an individual, and immobilizing said epidermal or skin equivalent at the site of contact.

29. The method of claim 28, wherein said outer root sheath cells are autologous cells derived from the individual who will subsequently undergo treatment for a skin defect.  
5
30. The method of claim 28, wherein said outer root sheath cells are homologous cells.
31. The method of claim 28, wherein the culture density of said keratinocyte precursor cells is between about  $3 \times 10^4$  cells/cm<sup>2</sup> and about  $1 \times 10^5$  cells/cm<sup>2</sup>.  
10
32. The method of claim 28, wherein the culture density of said growth-arrested/limited feeder cells on said microporous membrane is between about  $1 \times 10^4$  cells/cm<sup>2</sup> and about  $5 \times 10^4$  cells/cm<sup>2</sup>.  
15
33. The method of claim 28, wherein said growth-arrested/limited feeder cells are banked or immortalized cells.
34. The method of claim 28, wherein said primary and organotypic cultures utilize autologous or homologous human serum.  
20
35. The method of claims 28, wherein said primary and organotypic cultures utilize autologous or homologous human serum in a concentration of less than about 5%.  
25
36. The method of claim 28, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing only homologous or autologous biological supplements.
37. The method of claim 28, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing only homologous or autologous releasates from blood components.  
30

38. The method of claims 28, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing only homologous or autologous releasates from blood components at a concentration of about 0.1% to about 20%.  
5
39. The method of claim 28, wherein said epidermal equivalents are coated on their top or cornified side with a fibrin glue.
- 10 40. The method of claims 28, wherein said epidermal equivalents are coated on their top or cornified side with a carrier membrane.
- 15 41. The method of claims 28, wherein the graft thickness for said epidermal equivalents is 50-150 microns.
42. The method of claims 28, wherein the graft thickness for said complex skin equivalents does not exceed 0.4 mm.
- 20 43. The method of claim 28, wherein said microporous membrane is coated by one or ore extracellular matrix substances selected from a group consisting of: fibrin, fibronectin, collagens, laminins and hyaluronan.
- 25 44. The method of claims 28, wherein said microporous membrane possesses a growth-arrested/limited feeder cell system on its undersurface with said feeder cells of at least one type of cell selected from the group comprising human dermal fibroblasts, epidermal cells, mesenchymal cells, neuronal cells and endothelial cells.
- 30 45. The method of claim 28, wherein said carrier membrane is made from one or more types of materials selected from the group comprising polyester, PTFE, polyurethane, hyaluronic acid, polylactic acid, collagen, or a silicone or vaseline gauze dressing.

46. The method of claim 28, wherein the size of said epidermal equivalent is selected from the group consisting of 1.0 cm, 1.5 cm, 2.0 cm, and 2.5 cm in diameter.
- 5  
47. A method of shipping or transporting epidermal equivalents comprising:
  - (a) detaching said epidermal equivalents from a culture medium, and
  - (b) transferring said epidermal equivalents onto a transport medium.
- 10  
48. The method of claim 47, wherein said epidermal equivalents are coated on their top or cornified side with a carrier membrane.
49. The method of claim 48, wherein said epidermal equivalents are further sealed and shipped for future use in grafting.
- 15  
50. The method of claim 47, wherein said transport medium comprises a solidified or gelled medium.
51. The method of claim 50, wherein said solidified or gelled medium is selected from the group consisting of agarose, methyl cellulose, or another gelifying substance.

20



## ATENT COOPERATION TF TY

From the INTERNATIONAL BUREAU

PCT

## NOTIFICATION OF ELECTION

(PCT Rule 61.2)

Date of mailing (day/month/year) 09 April 2001 (09.04.01)	To:  Commissioner US Department of Commerce United States Patent and Trademark Office, PCT 2011 South Clark Place Room CP2/5C24 Arlington, VA 22202 ETATS-UNIS D'AMERIQUE  in its capacity as elected Office
International application No. PCT/IB00/01076	Applicant's or agent's file reference 17811-014
International filing date (day/month/year) 20 July 2000 (20.07.00)	Priority date (day/month/year) 20 July 1999 (20.07.99)
Applicant  HUNZIKER, Thomas et al	

1. The designated Office is hereby notified of its election made:

in the demand filed with the International Preliminary Examining Authority on:

19 February 2001 (19.02.01)

in a notice effecting later election filed with the International Bureau on:

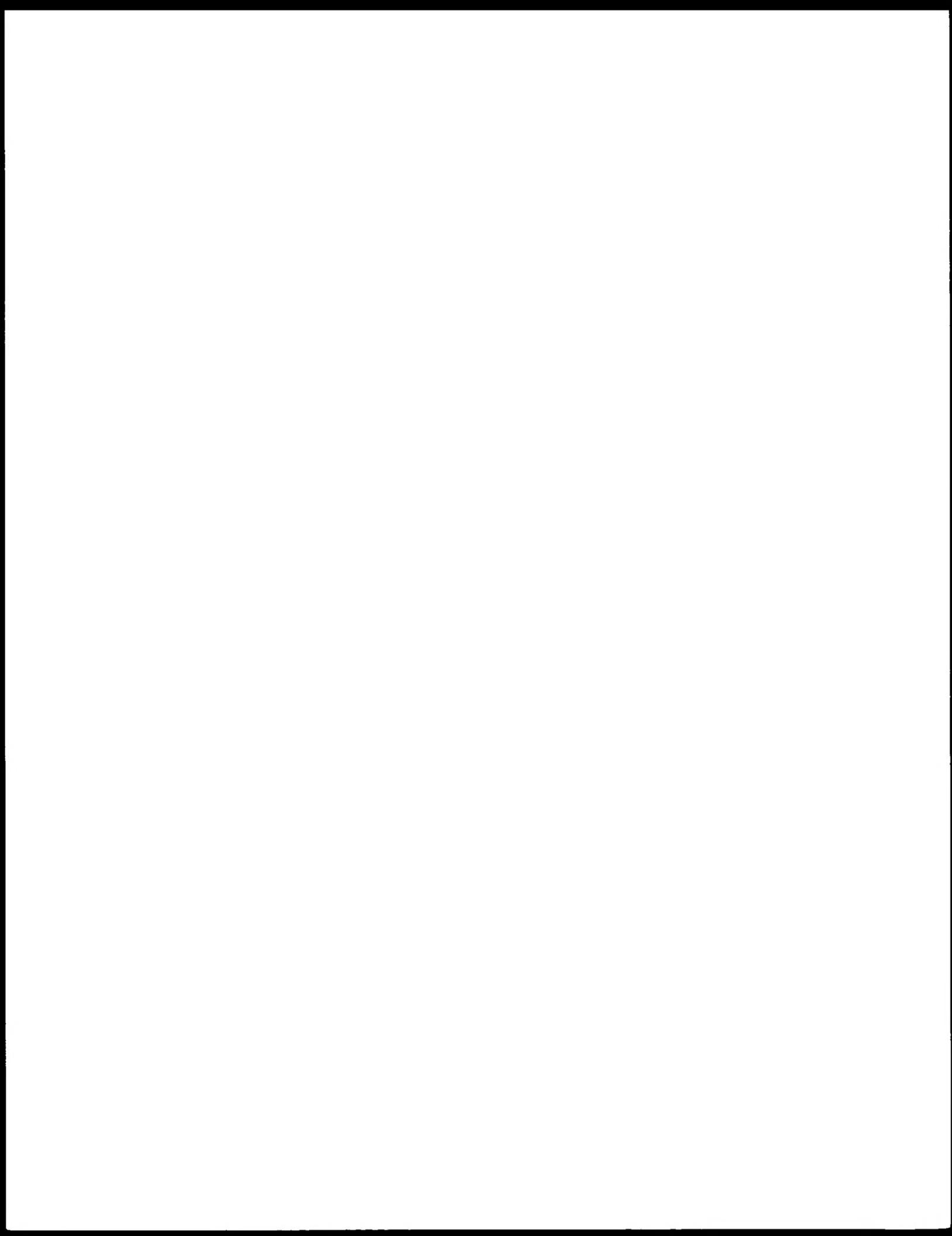
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2. The election  was

was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland  Facsimile No.: (41-22) 740 14.35	Authorized officer  Pascal Piriou  Telephone No.: (41-22) 338.83.38
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## PATENT COOPERATION TREATY

PCT

NOTIFICATION OF THE RECORDING  
OF A CHANGE(PCT Rule 92bis.1 and  
Administrative Instructions, Section 422)

From the INTERNATIONAL BUREAU

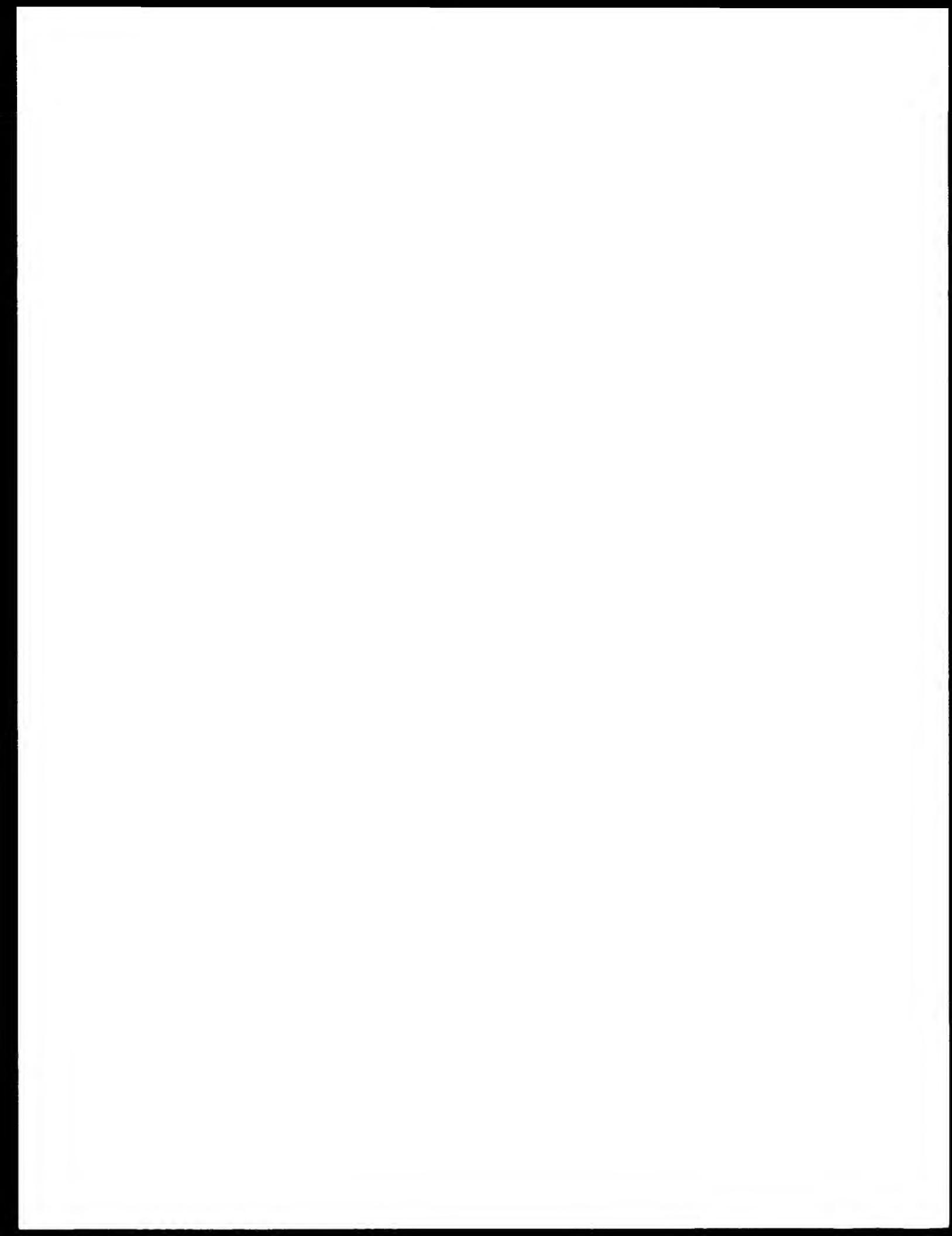
To:

MACLEAN, Martin, Robert  
 Mathys & Squire  
 100 Gray's Inn Road  
 London WC1X 8AL  
 ROYAUME-UNI

Date of mailing (day/month/year) 27 November 2000 (27.11.00)	
Applicant's or agent's file reference 17811-014	IMPORTANT NOTIFICATION
International application No. PCT/IB00/01076	International filing date (day/month/year) 20 July 2000 (20.07.00)

1. The following indications appeared on record concerning: <input type="checkbox"/> the applicant <input type="checkbox"/> the inventor <input checked="" type="checkbox"/> the agent <input type="checkbox"/> the common representative				
Name and Address     	State of Nationality		State of Residence	
	Telephone No.			
	Facsimile No.			
	Teleprinter No.			
2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning: <input type="checkbox"/> the person <input type="checkbox"/> the name <input type="checkbox"/> the address <input type="checkbox"/> the nationality <input type="checkbox"/> the residence				
Name and Address MACLEAN, Martin, Robert Mathys & Squire 100 Gray's Inn Road London WC1X 8AL United Kingdom	State of Nationality		State of Residence	
	Telephone No. +44 (0)20 7830 0000			
	Facsimile No. +44 (0)20 7830 0001			
	Teleprinter No.			
3. Further observations, if necessary: <b>APPOINTMENT OF AGENT.</b>				
4. A copy of this notification has been sent to: <input checked="" type="checkbox"/> the receiving Office <input type="checkbox"/> the designated Offices concerned <input checked="" type="checkbox"/> the International Searching Authority <input type="checkbox"/> the elected Offices concerned <input type="checkbox"/> the International Preliminary Examining Authority <input type="checkbox"/> other:				

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland  Facsimile No.: (41-22) 740.14.35	Authorized officer  Peggy Steunenberg  Telephone No.: (41-22) 338 83.38
---	---



From the  
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

MACLEAN, Martin, Robert  
Mathys & Squire  
100 Gray's Inn Road  
London WC1X 8AL  
GRANDE BRETAGNE

RECEIVED	
MATHYS & SQUIRE	
25 OCT 2001	
REPLY DATE	
DIARY ENTRY	

PCT

NOTIFICATION OF TRANSMITTAL OF  
THE INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT

(PCT Rule 71.1)

Applicant's or agent's file reference  
GWS/MRM/22559

**IMPORTANT NOTIFICATION**

International application No.  
PCT/IB00/01076

International filing date (day/month/year)  
20/07/2000

Priority date (day/month/year)  
20/07/1999

Applicant  
EPITECH S.A. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

**4. REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/

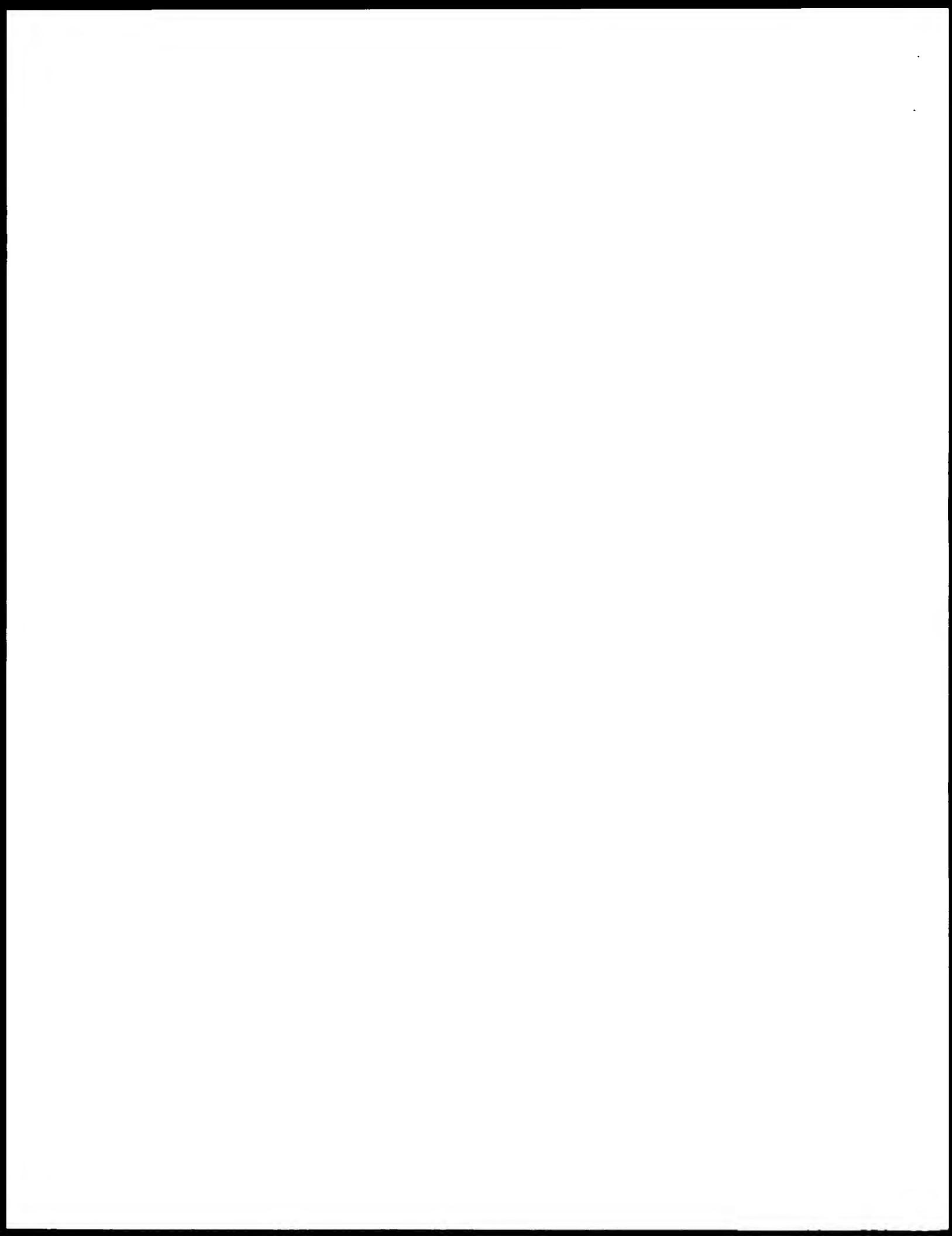
European Patent Office  
D-80298 Munich  
Tel. +49 89 2399 - 0 Tx: 523656 epmu d  
Fax: +49 89 2399 - 4465

Authorized officer

Hingel, W

Tel. +49 89 2399-8717





## PATENT COOPERATION TREATY

PCT

**INTERNATIONAL PRELIMINARY EXAMINATION REPORT**  
**(PCT Article 36 and Rule 70)**

Applicant's or agent's file reference  GWS/MRM/22559	<b>FOR FURTHER ACTION</b>	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No.  PCT/IB00/01076	International filing date (day/month/year)  20/07/2000	Priority date (day/month/year)  20/07/1999
International Patent Classification (IPC) or national classification and IPC  C12N5/00		
Applicant  EPITECH S.A. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 10 sheets, including this cover sheet.

- This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

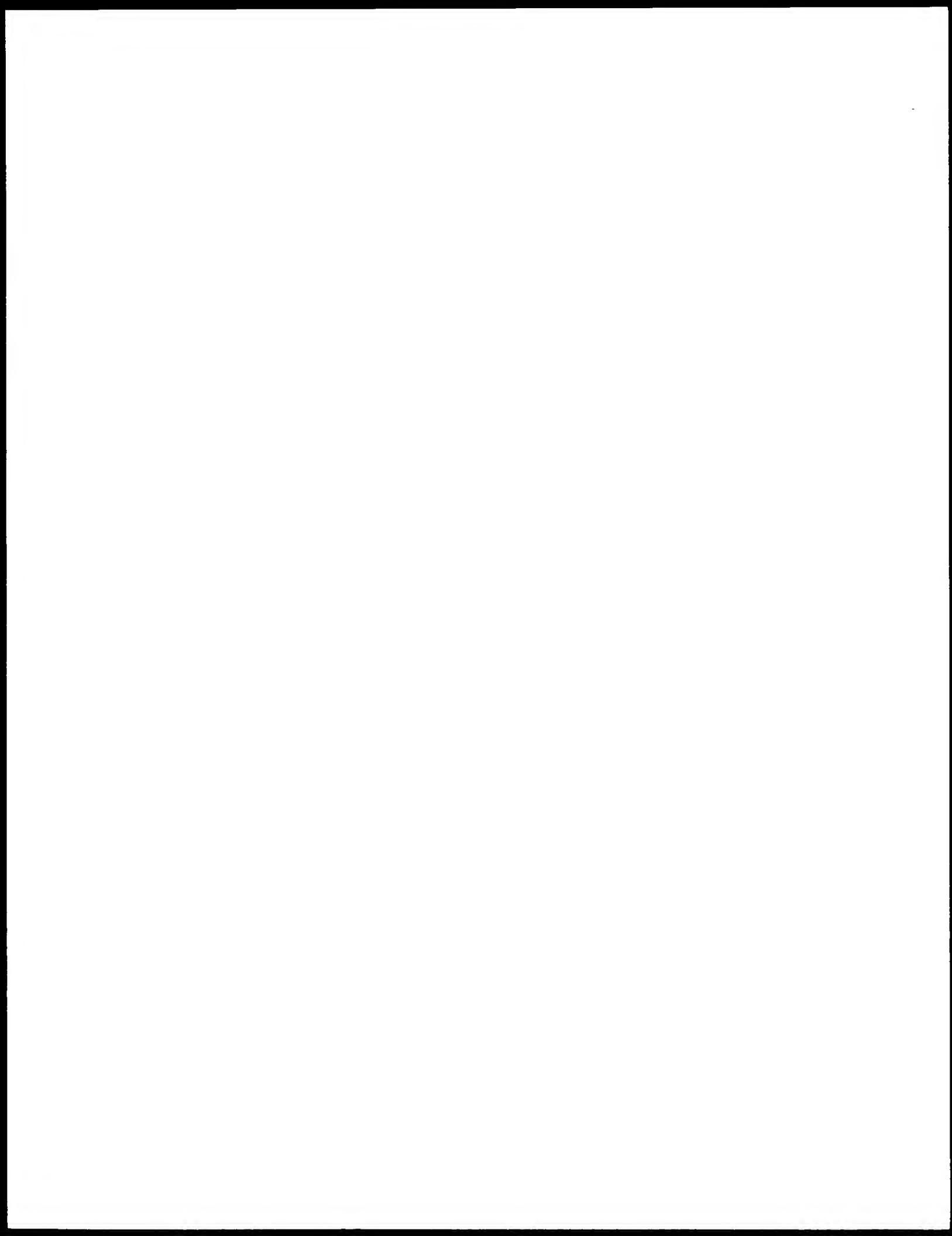
These annexes consist of a total of 9 sheets.

3. This report contains indications relating to the following items:

- I    Basis of the report
- II    Priority
- III    Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV    Lack of unity of invention
- V    Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI    Certain documents cited
- VII    Certain defects in the international application
- VIII    Certain observations on the international application

Date of submission of the demand  19/02/2001	Date of completion of this report  23.10.2001
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer  Nichogiannopoulou, A  Telephone No. +49 89 2399 8054





I. Basis of the report

1. With regard to the **elements** of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):

Description, pages:

1,2,4-26 as originally filed

3,3a as received on 26/09/2001 with letter of 25/09/2001

Claims, No.:

1-51 as received on 26/09/2001 with letter of 25/09/2001

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

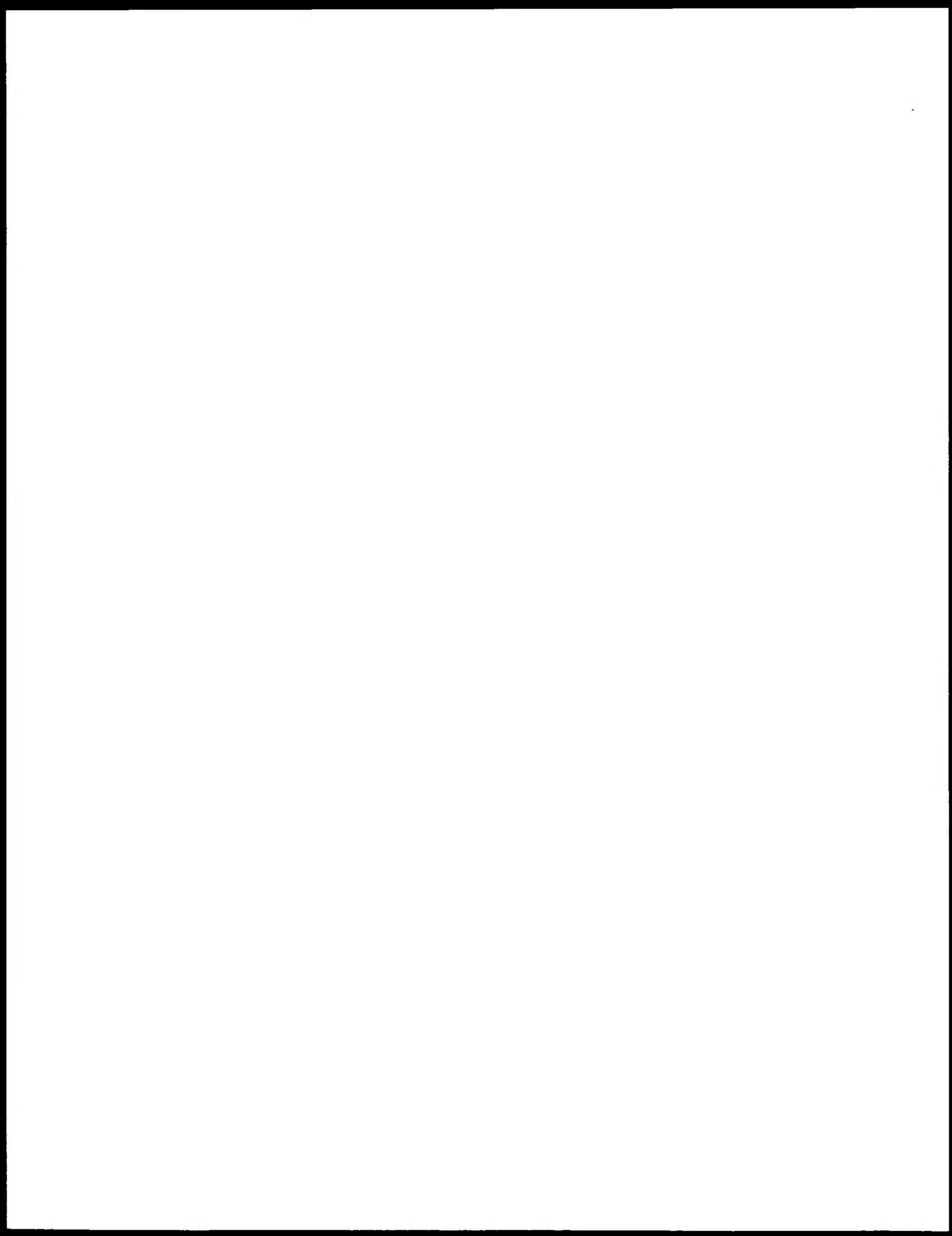
- the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- the language of publication of the international application (under Rule 48.3(b)).
- the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- contained in the international application in written form.
- filed together with the international application in computer readable form.
- furnished subsequently to this Authority in written form.
- furnished subsequently to this Authority in computer readable form.
- The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- the description, pages:
- the claims, Nos.:
- the drawings, sheets:



5.  This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

*(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)*

6. Additional observations, if necessary:

**II. Priority**

1.  This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

copy of the earlier application whose priority has been claimed.

translation of the earlier application whose priority has been claimed.

2.  This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

**see separate sheet**

**III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

the entire international application.

claims Nos. 1-46.

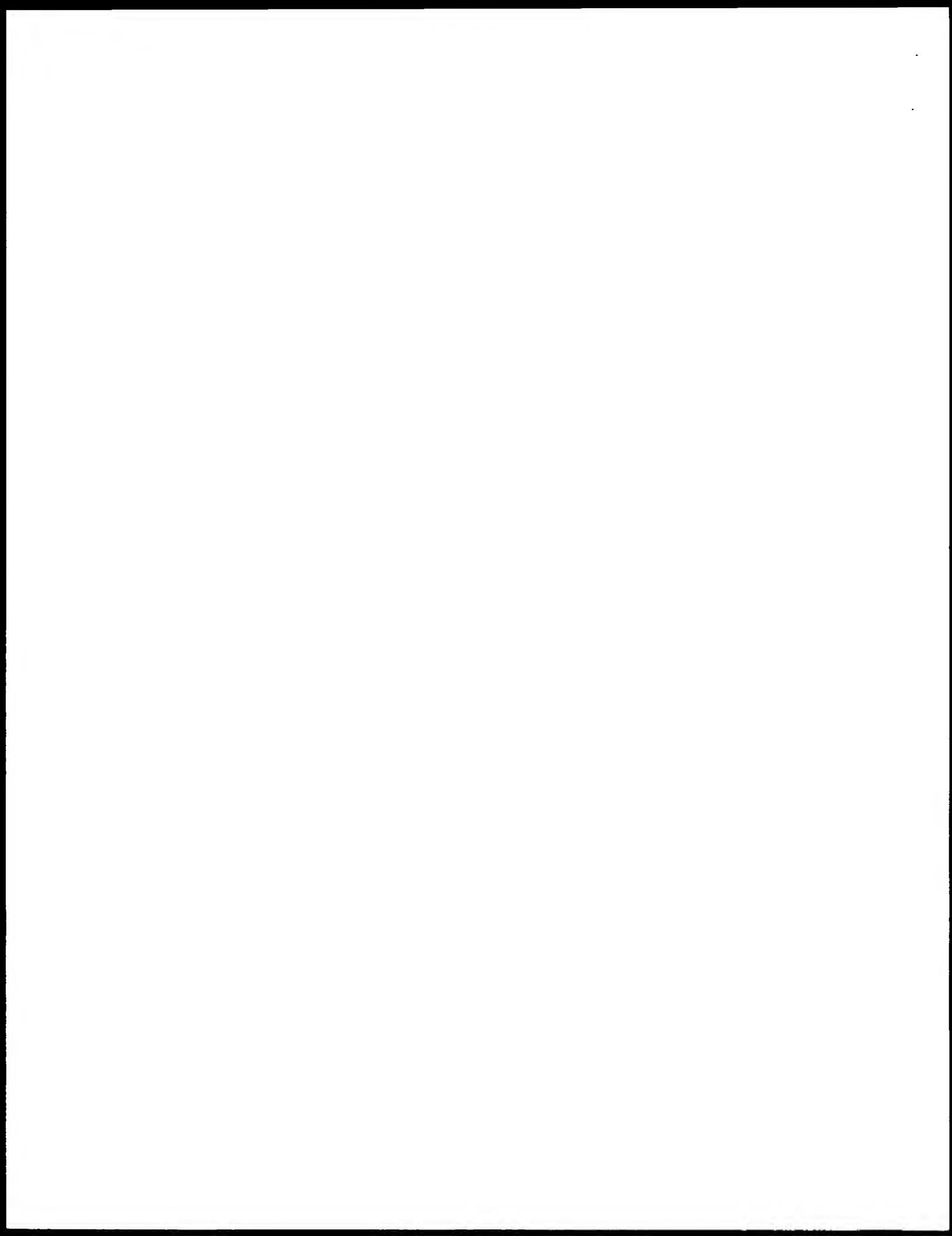
because:

the said international application, or the said claims Nos. 1-46 relate to the following subject matter which does not require an international preliminary examination (*specify*):  
**see separate sheet**

the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

no international search report has been established for the said claims Nos. .



2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

- the written form has not been furnished or does not comply with the standard.
- the computer readable form has not been furnished or does not comply with the standard.

**IV. Lack of unity of invention**

1. In response to the invitation to restrict or pay additional fees the applicant has:

- restricted the claims.
- paid additional fees.
- paid additional fees under protest.
- neither restricted nor paid additional fees.

2.  This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

- complied with.
- not complied with for the following reasons:

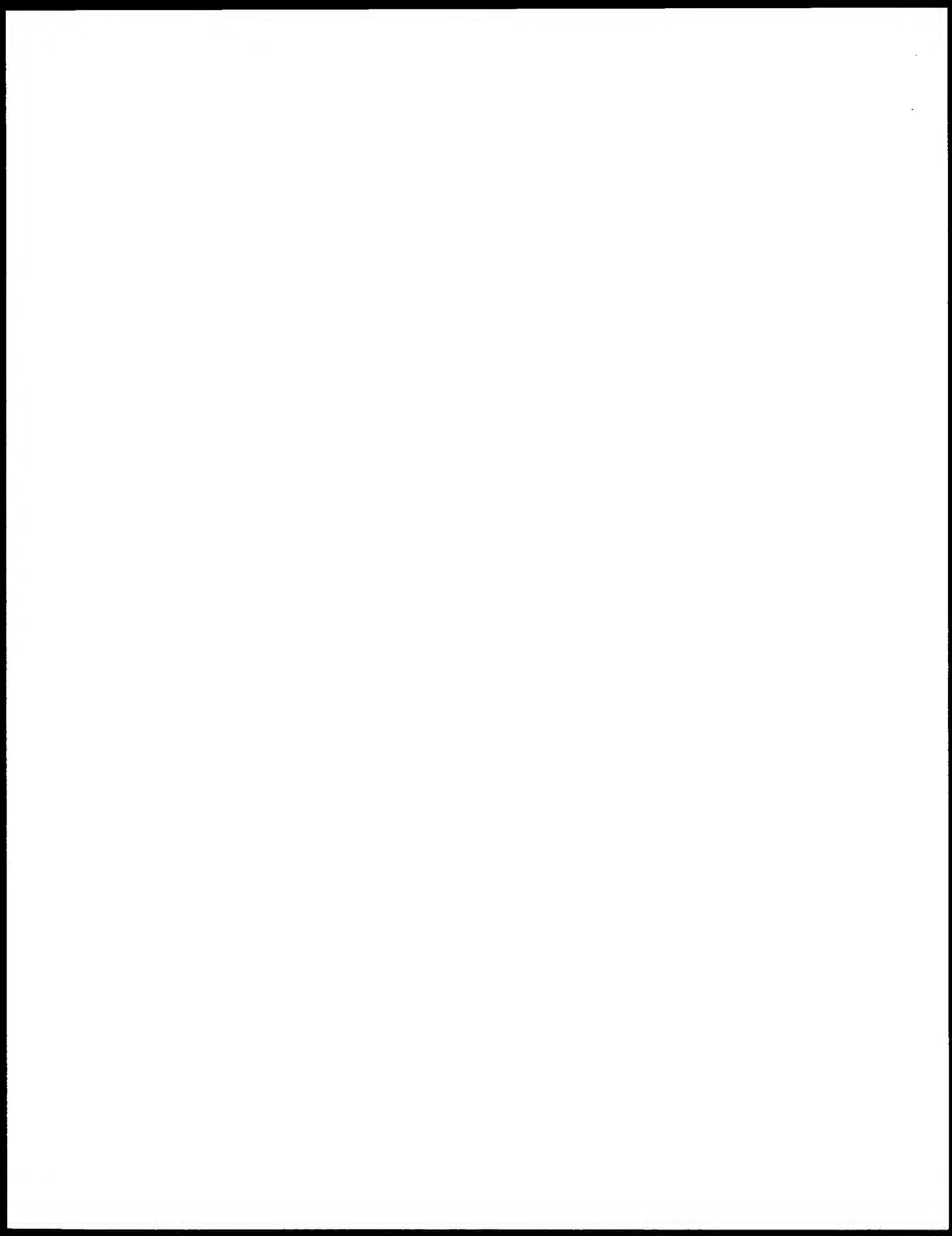
4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

- all parts.
- the parts relating to claims Nos. 1-46.

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

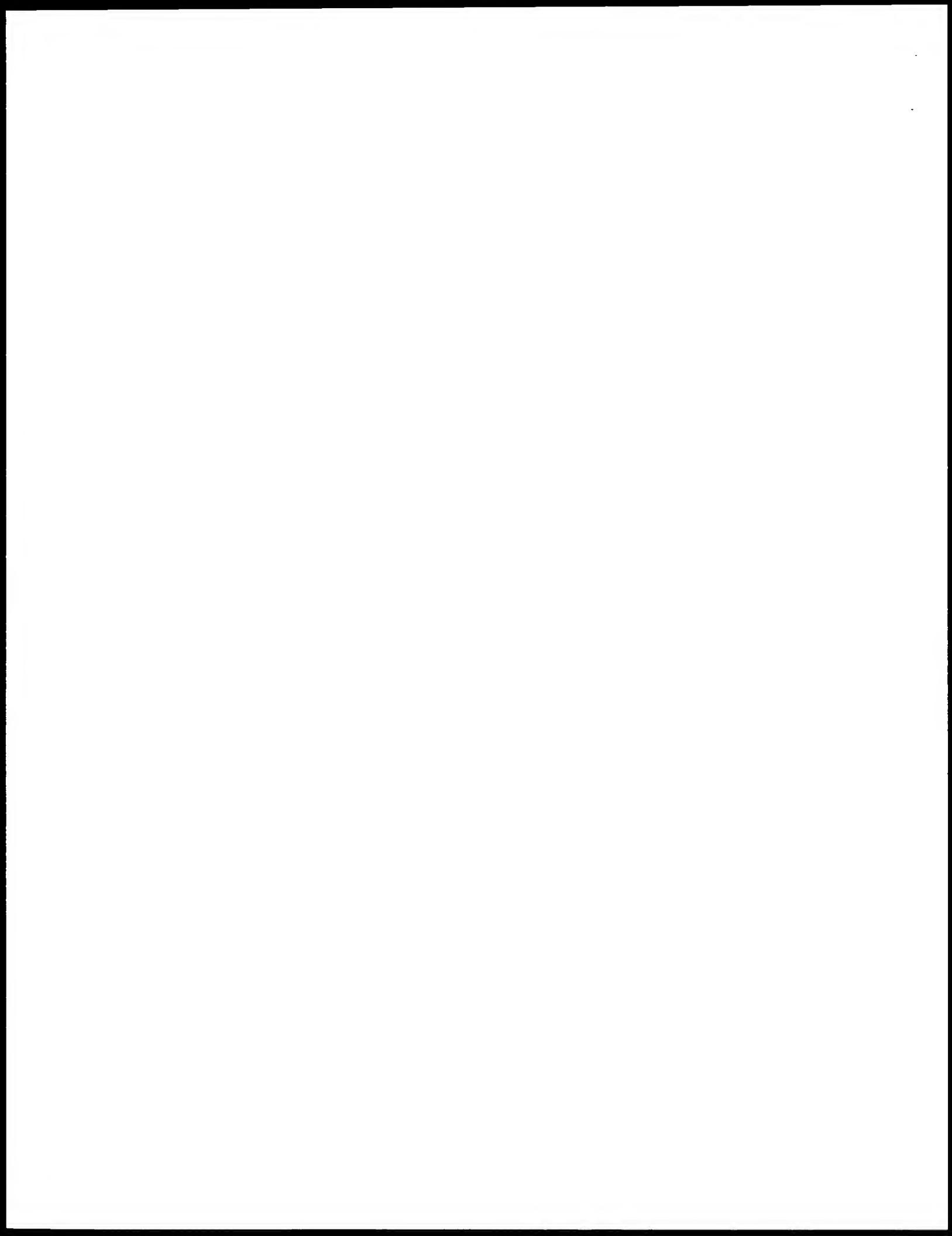
Novelty (N)	Yes:	Claims 1-46
	No:	Claims
Inventive step (IS)	Yes:	Claims 8, 13-27, 39-46
	No:	Claims 1-7, 9-12, 28-38
Industrial applicability (IA)	Yes:	Claims see box III
	No:	Claims



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/IB00/01076

2. Citations and explanations  
**see separate sheet**



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

International Application No. PCT/IB00/01076

**Re Item I**

**Basis of the report**

1. The amendments filed with the letter of 25.09.2001 are formally allowable under Article 34(2)(b) PCT because they do not introduce subject-matter extending beyond the content of the application as filed.

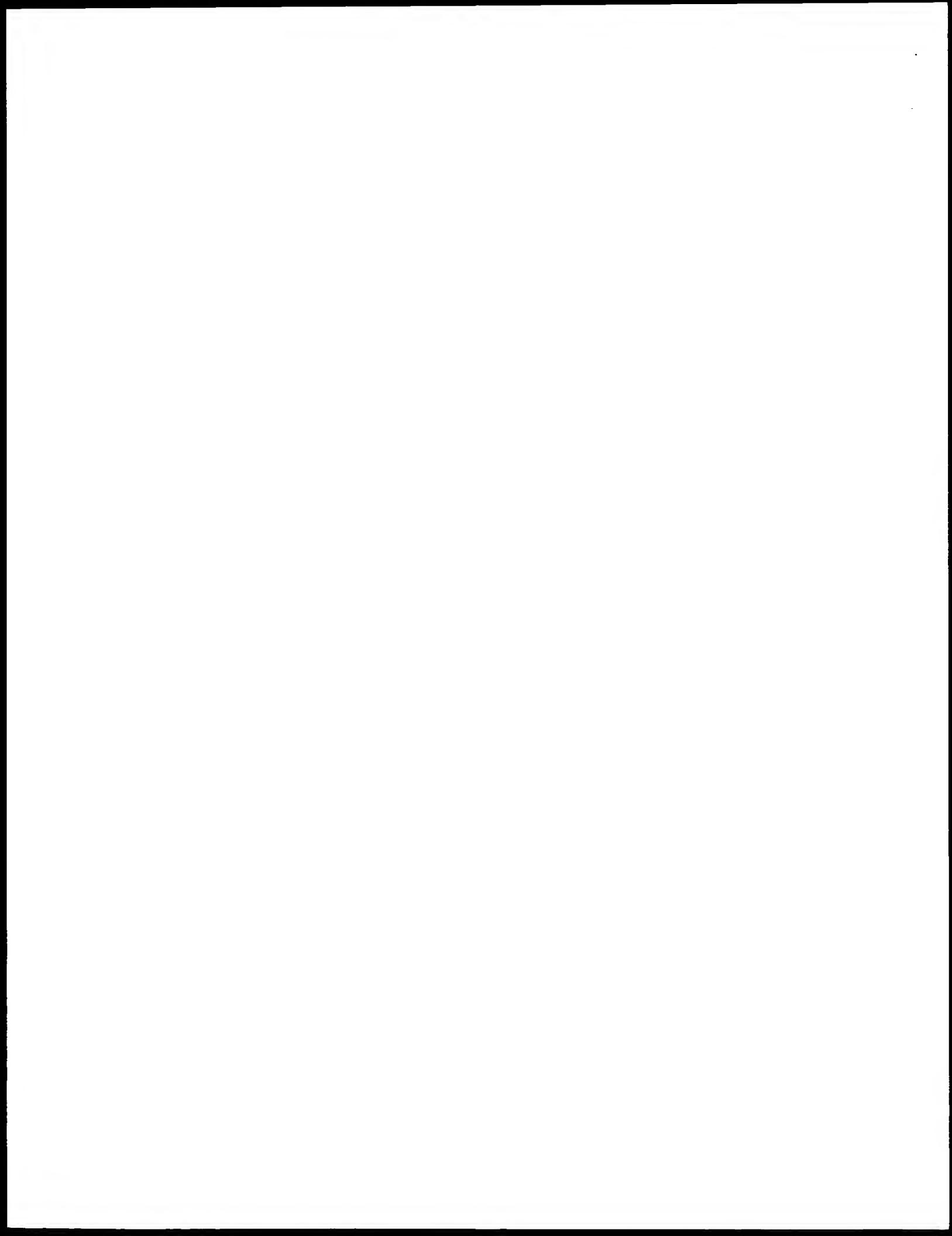
**Re Item II**

**Priority**

1. The following document was published prior to the international filing date but later than the priority date claimed (P-document):

P1: US-A-5 968 546 (BAUR MARCUS ET AL) 19 October 1999 (1999-10-19)

2. The priority document pertaining to the present application was not available at the time of establishing this report. Hence, the current assessment is based on the assumption that all claims enjoy priority rights from the filing date of the priority document (20.07.1999). If it later turns out that this assumption is incorrect, P1 will become relevant to the assessment of whether the present application satisfies the criteria set forth in Article 33(2) and (3) PCT.



Re Item III

**Non-establishment of opinion with regard to novelty, inventiveness and industrial applicability**

1. No meaningful examination could be performed for claims 1-46 for the following reason:

Claims 1-46 -as far as they concern *in vivo* methods- relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

Re Item IV

**Lack of unity of invention**

1. The present application addresses the generation of *in vitro* cultured epidermal equivalents and methods for their transport.

The generation of epidermal equivalents, in particular from outer root sheath cells, is widely known from the prior art (see Limat et al., 1996).

In the light of the prior art, the problems and corresponding solutions of the present application can be summarized as follows:

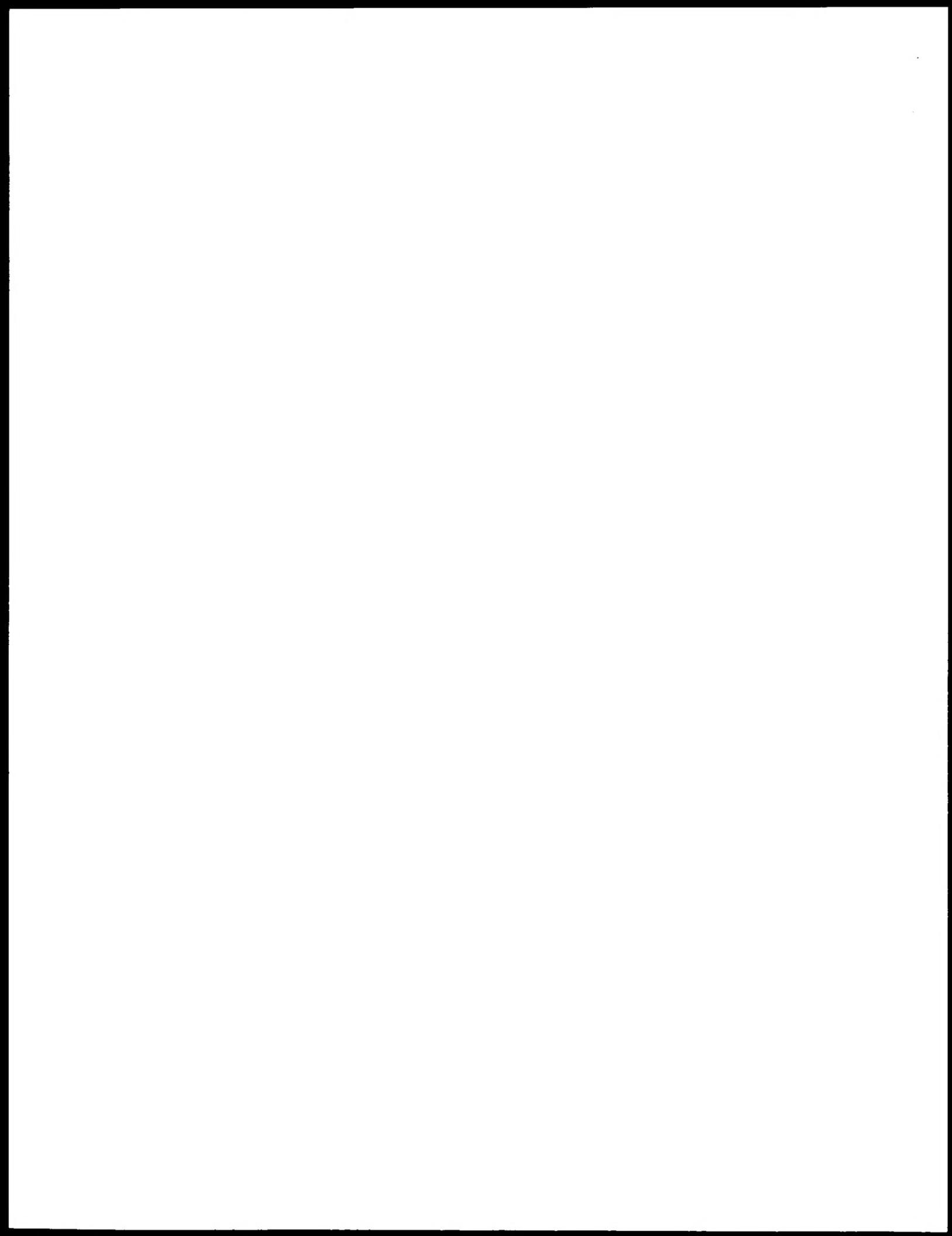
Problem I: Providing alternatives for the generation of epidermal equivalents from outer root sheath cells.

Solution 1: claims 1-3, 6, 9, 10 all completely and 4, 5, 7, 8, 12, 21, 28-46 all partially

A method for the generation of an epidermal equivalent from keratinocyte precursors from outer root sheath (ORS) cells by culture of anagen hair *in toto*.

Solution 2: claims 11, 13-15, 18, 19 all completely and 4, 5, 12, 16, 17, 24, 25, 34-38 all partially

A method for the generation of an epidermal equivalent from keratinocyte precursors from ORS cells by culturing in media



supplemented with less than 5% autologous or homologous human serum.

Solution 3: claims 20, 22, 23, 26, 27 all completely and 7, 8, 16, 17, 21, 24, 25, 28-46 all partially

A method for the generation of an epidermal equivalent from keratinocyte precursors from ORS cells wherein said epidermal equivalent is coated on its top side with a fibrin glue.

Problem II: Providing a method for the transport of cultured epidermal equivalents.

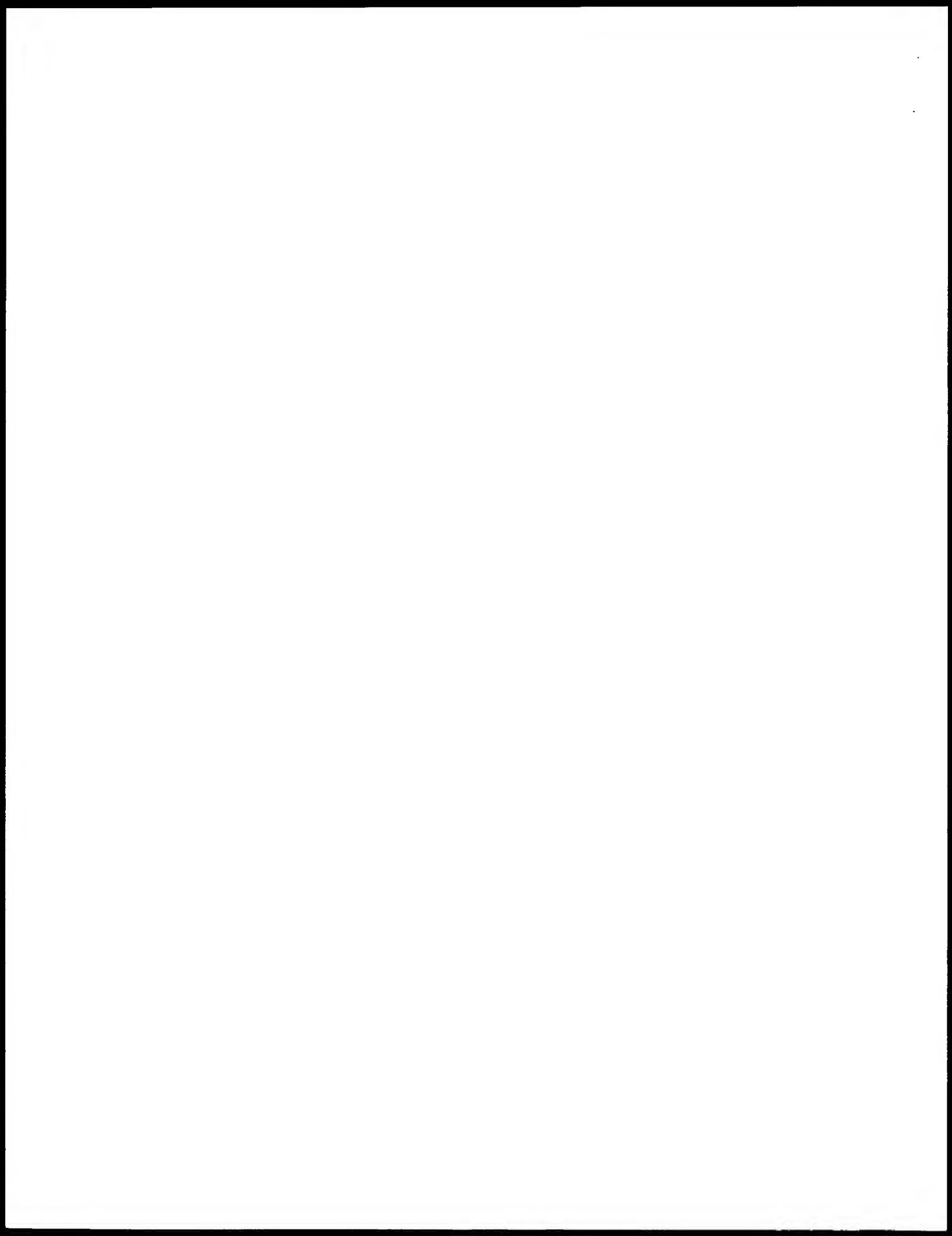
Solution 4: claims 47-51

A method of detaching epidermal equivalents and transferring them into an appropriate medium

Solutions 1-3 of problem I and solution 4 of problem II represent *a priori* distinct problems and their respective solutions as brought forward in the present application are not so linked as to form a single inventive concept. Since the special technical features outlined above are not the same or corresponding, the requirement of unity in the sense of Rule 13.2 PCT is not fulfilled. Consequently there is lack of unity, and there are four different inventions not belonging to a common inventive concept.

#### R Item V

**Reasoned statement under Article 35(2) with regard to novelty, inventiveness step or industrial applicability; citations and explanations supporting such statement**



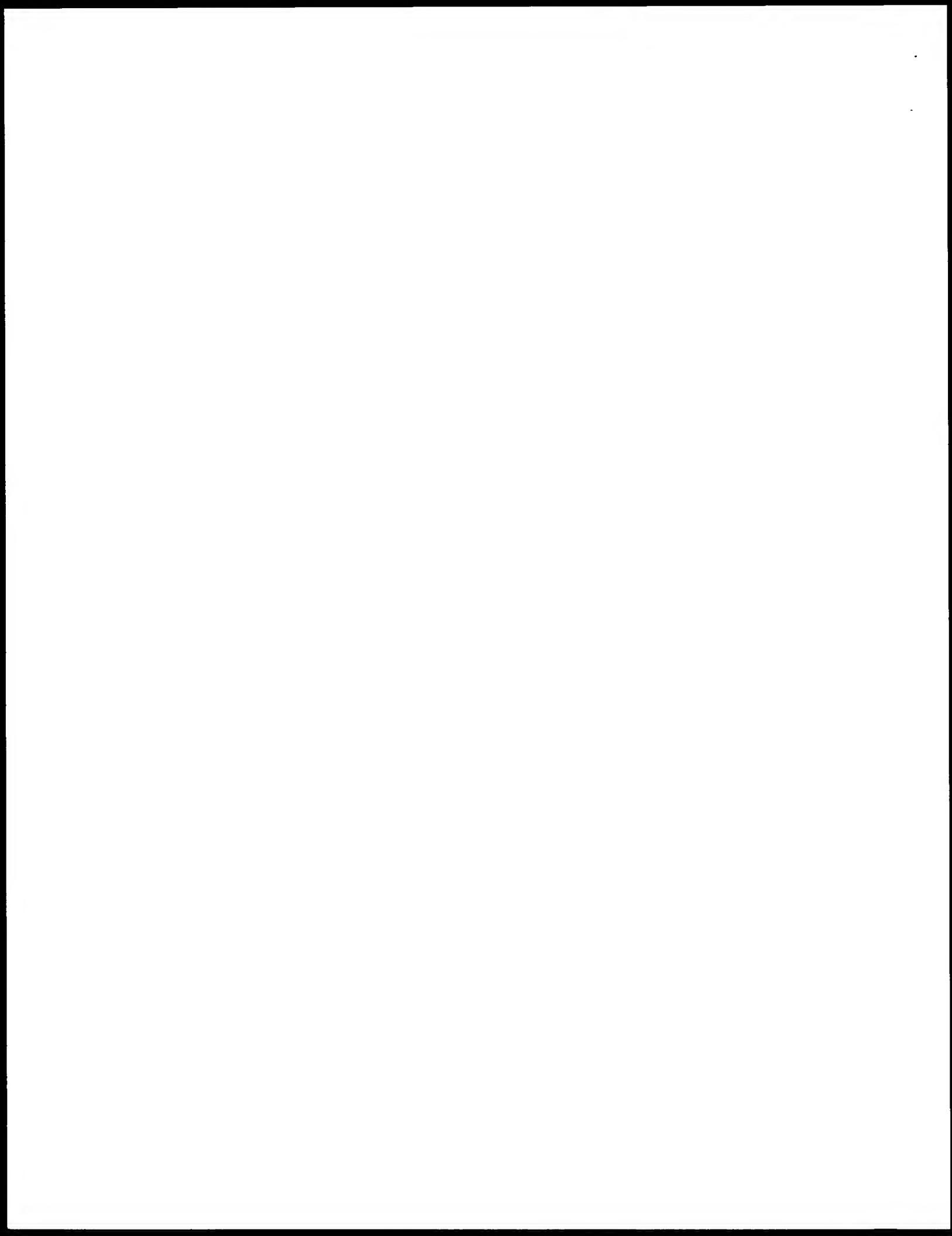
1. Reference is made to the following documents:

- D1: DE 196 51 992 A (TOLOCZYKI CHRISTIAN DR) 25 June 1998 (1998-06-25)
- D2: LENOIR-VIALE M -C ET AL: "Epidermis reconstructed from the outer root sheath of human hair follicle: Effect of retinoic acid." ARCHIVES OF DERMATOLOGICAL RESEARCH, vol. 285, no. 4, 1993, pages 197-204, XP000960515 ISSN: 0340-3696
- D3: LIMAT ALAIN ET AL: "Successful treatment of chronic leg ulcers with epidermal equivalents generated from cultured autologous outer root sheath cells." JOURNAL OF INVESTIGATIVE DERMATOLOGY, vol. 107, no. 1, 1996, pages 128-135, XP000960501 ISSN: 0022-202X

2. The present application discloses that skin equivalents can be obtained in organotypic cultures from outer root sheath (ORS) cells derived from culturing anagen or growing hair *in toto*. The culture conditions include (i) culturing of the hair *in toto* upon microporous membranes carrying human fibroblast feeder cells at their under-surface, (ii) seeding of the thus obtained ORS cells upon a modified microporous membrane carrying feeder cells at their under-surface, where they differentiate to form an epidermal equivalent, (iii) using a reduced concentration of allogenic or homologous serum and substances isolated from blood components, (iv) devising a method to transport the epidermal equivalents that reduces the probability of mechanical damage.

3. **Novelty (Article 33(2) PCT)**

Each of the independent claims is directed to methods relating to the culture of intact hair follicles of an anagenic hair to obtain outer root sheath cells. The IPEA notes that this feature is not disclosed in the prior art and thus claims 1-46 are novel under the terms of Article 33(2) PCT.



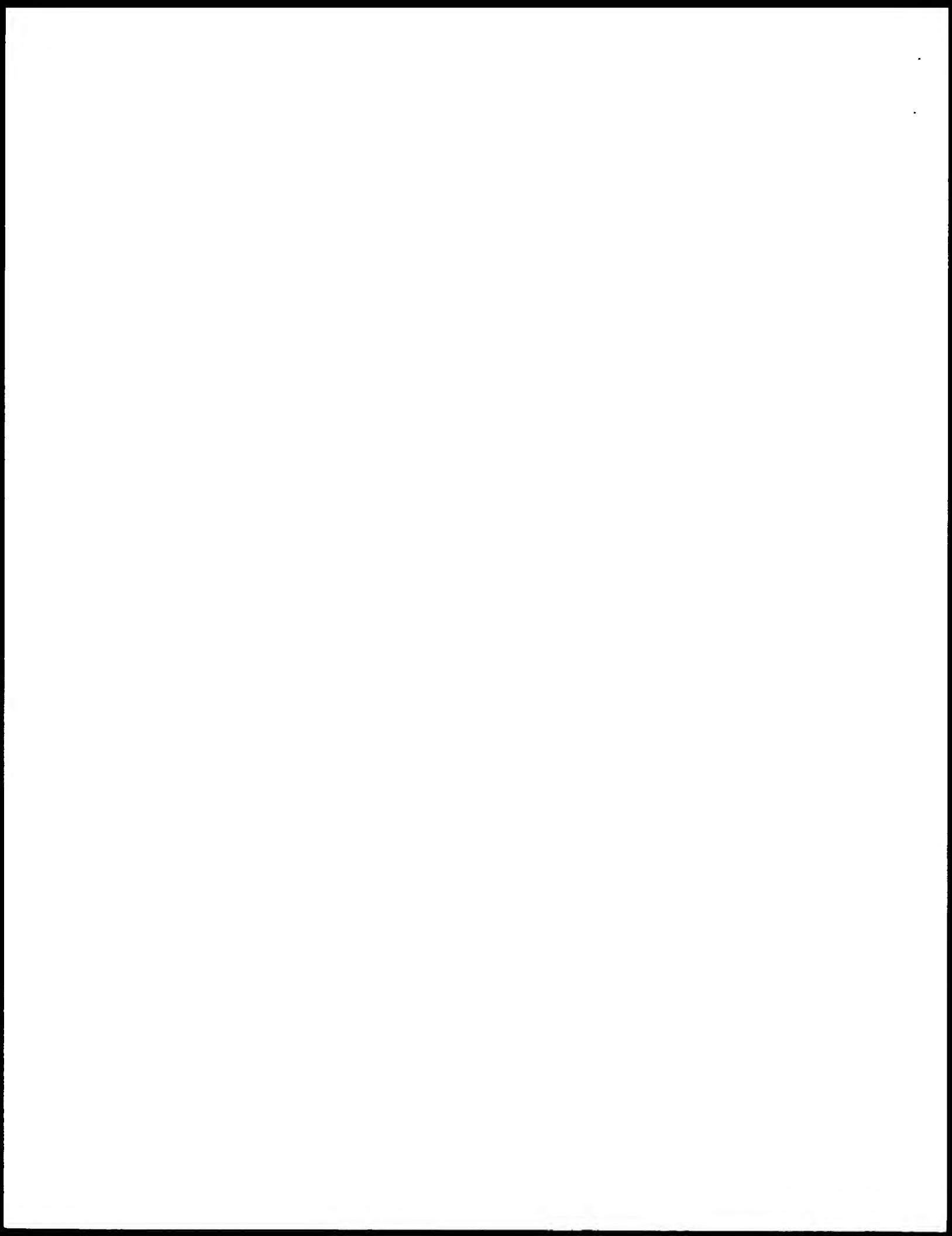
4. **Inventive step** (Article 33(3) PCT)

**D1** is a patent application by the same inventors on the use of 10-15% (functioning range 3-60%) autologous or homologous serum in the culture of dermal equivalents from ORS cells (column 3, line 24). A further increase in cell growth was achieved by the addition of 0.05-1% autologous or homologous platelet released peptides (=releasates) (column 3, lines 56-65). The dermal equivalents were seeded on hyaluronic acid membranes or other biodegradable material prior to transplantation to optimise handling (column 4, lines 26-36).

**D2** discloses the culture of ORS cells from plucked anagen hair *in toto*, without prior removal of the bulbs (i.e. intact hair follicles). Specifically, lines 6-7 of column 1 on page 198 states that "in some experiments, the bulbs were removed using scissors", implicating that in others, the bulbs were not removed and the intact hair follicle was cultured. ORS cells are grown on dead de-epidermised dermis as opposed to human dermal fibroblasts. **D3** is a publication by the inventors on basically the same technique as the application except (i) the culturing of hair *in toto*, (ii) the culturing in <5% human serum and (iii) the coating with fibrin glue. At the relevant filing date of the present application, the skilled person in search for optimised treatments of patients in need of skin grafts would have combined the teaching on *in toto* hair culture of **D2**, with the teaching on the use of human serum and releasates of **D1** and the general teachings of **D3**, to arrive at the subject-matter of present claims 1-7, 9-12, and 28-38. Said claims are thus found to lack an inventive step under the terms of Article 33(3) PCT.

5. **Industrial applicability** (Article 33(4) PCT)

The subject-matter of claims on which an opinion can be formed (see item III) appears to be industrially applicable under the terms of Article 33(4) PCT.



few, noncornified cell layers (Hetton *et al.*, 14 J. Am. Acad. Dermatol. 399-405 (1986); Leigh & Purkis, 11 Clin. Exp. Dermatol. 650-652 (1986); Leigh *et al.*, 117 Brit. J. Dermatol. 591-597 (1987); Harris *et al.*, 18 Clin. Exp. Dermatol. 417-420 (1993)), trypsinized single cells attached to collagen-coated dressings (Brysk *et al.*, 25 J. Am. Acad. Dermatol. 238-244 (1991)), skin equivalents (Mol *et al.*, 24 J. Am. Acad. Dermatol. 77-82 (1991)) has yet to be convincingly documented within the scientific literature. The same lack of quantitative findings also holds true for various reports on the grafting of freshly isolated, autologous interfollicular keratinocytes (Hunyadi *et al.*, 14 J. Dermatol. Surg. Oncol. 75-78 (1988)) or ORS cells (Moll *et al.*, 46 Hautarzt 548-552 (1995)) fixed to the wound bed by the use of a fibrin glue.

However, it should be noted that the disadvantages of the bovine serum used during cultivation of the keratinocytes may contribute to reduced "take" rate, due to the fact that it resists in keratinocytes (*see e.g.*, Johnson *et al.*, 11 J. Burn Care Rehab. 504-509 (1990)).

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SUMMARY OF THE INVENTION

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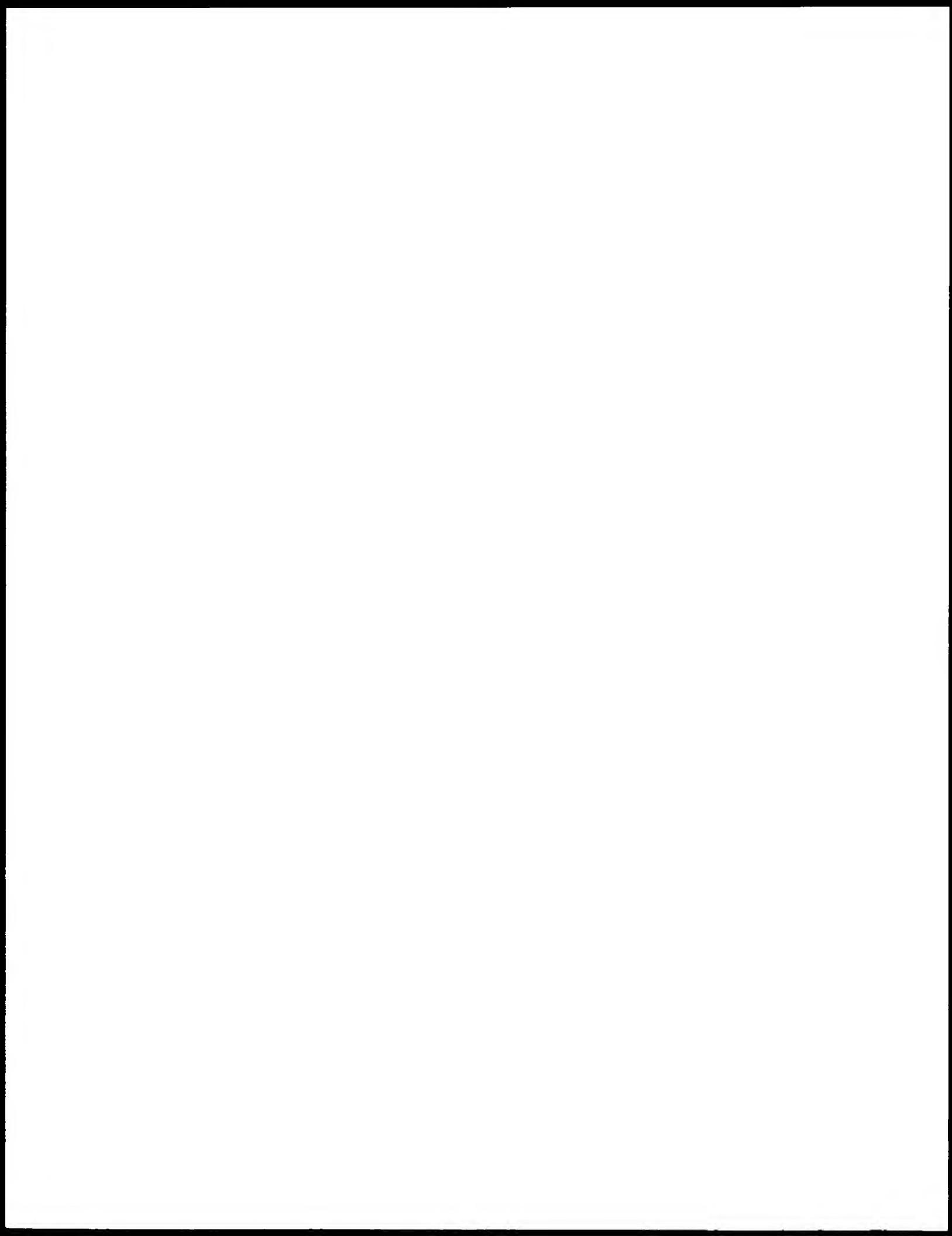
20

Prior to the disclosure of the present invention herein, the standard methodology for the generation of a primary culture of ORS keratinocytes consisted of the plucking of an anagen (*i.e.*, growing hair shaft) hair followed by a careful microscopic dissection to remove the hair bulbs and the infundibular hair shaft. The resulting outer root sheath was then placed on the culture insert for initiation of the primary keratinocyte culture. However, numerous subsequent studies (approximately 200), wherein the anagen hair was placed directly on the culture insert without performing the initial micro-dissection to remove the hair bulbs and the infundibular hair shaft, have demonstrated that such tedious and time-consuming dissection of the plucked anagen hair was not required. This has served to markedly simplify the handling process, reduce the risk for contamination, and resulted in more efficient initiation of keratinocyte cell plating.

25

Accordingly, it is an object of the present invention to provide improved and simplified methods for the generation of keratinocytes or keratinocyte precursors from outer root sheath cells (ORS cells) in fully defined culture conditions for the treatment of various types of skin defects (*e.g.*, chronic wounds such as leg ulcers, diabetic

30



DE-A-19651992 describes the culture of outer root sheath cells in 10-15% autologous or homologous serum to produce dermal equivalents. The dermal equivalents may be seeded on hyaluronic acid membranes or other biodegradable material prior to transplantation in order to optimise handling.

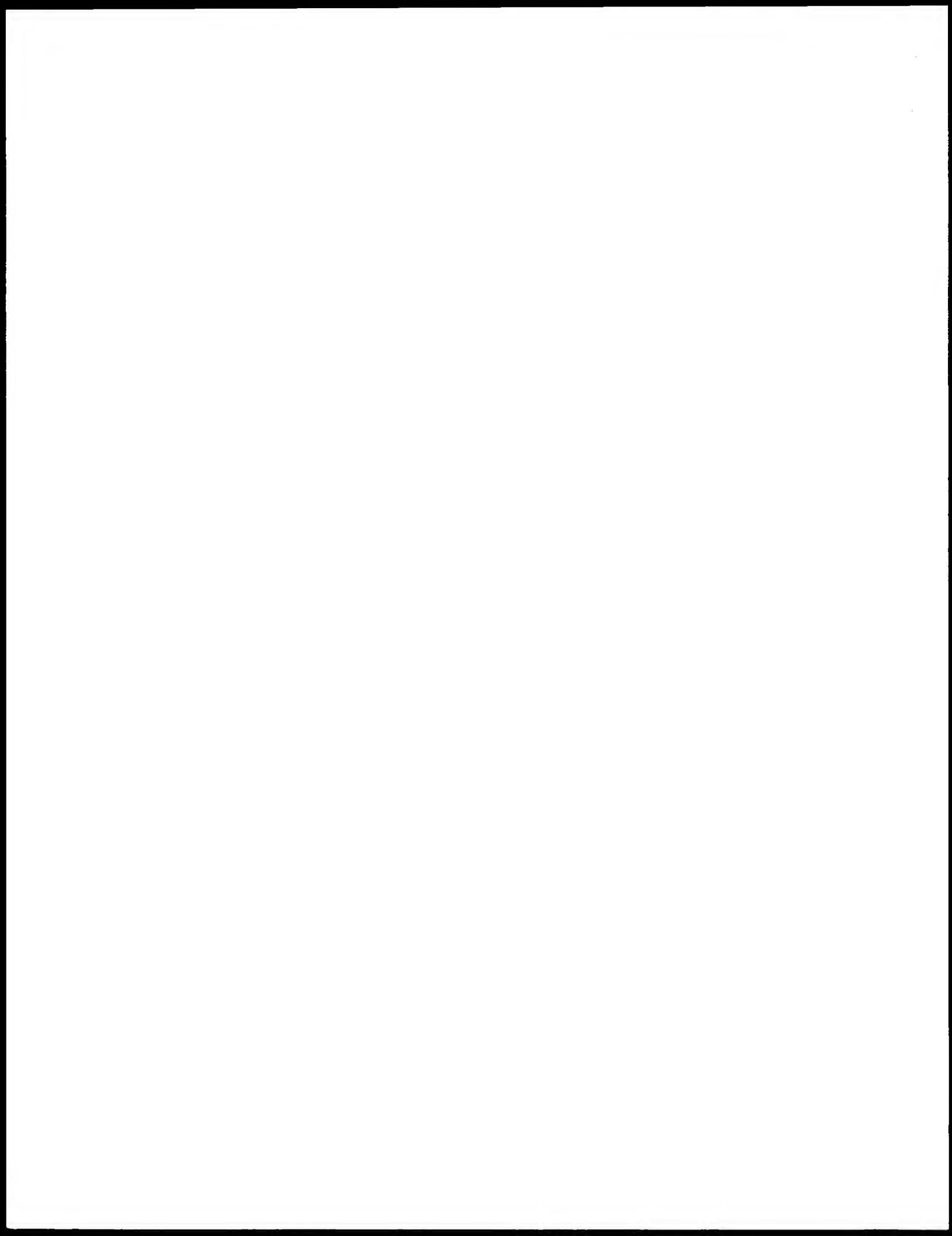
5 Lenoir-Viale, M. C. (Arch. Dermatol. Res. 1993, 285: pages 197-204) describes the *in vitro* preparation of a reconstructed epidermis from the outer root sheath of human hair follicles. The reconstructed epidermis is described as a valuable and promising tool for pharmacological studies and may represent a model of wound-healing.

10 Limat, A. (J. of Investigative Dermatology 2000, Nov. 7, pages 128-134) describes the culturing of hair follicles (hair bulbs and infundibular parts removed) to generate epidermal equivalents and the use thereof for treating chronic leg ulcers.

#### SUMMARY OF THE INVENTION

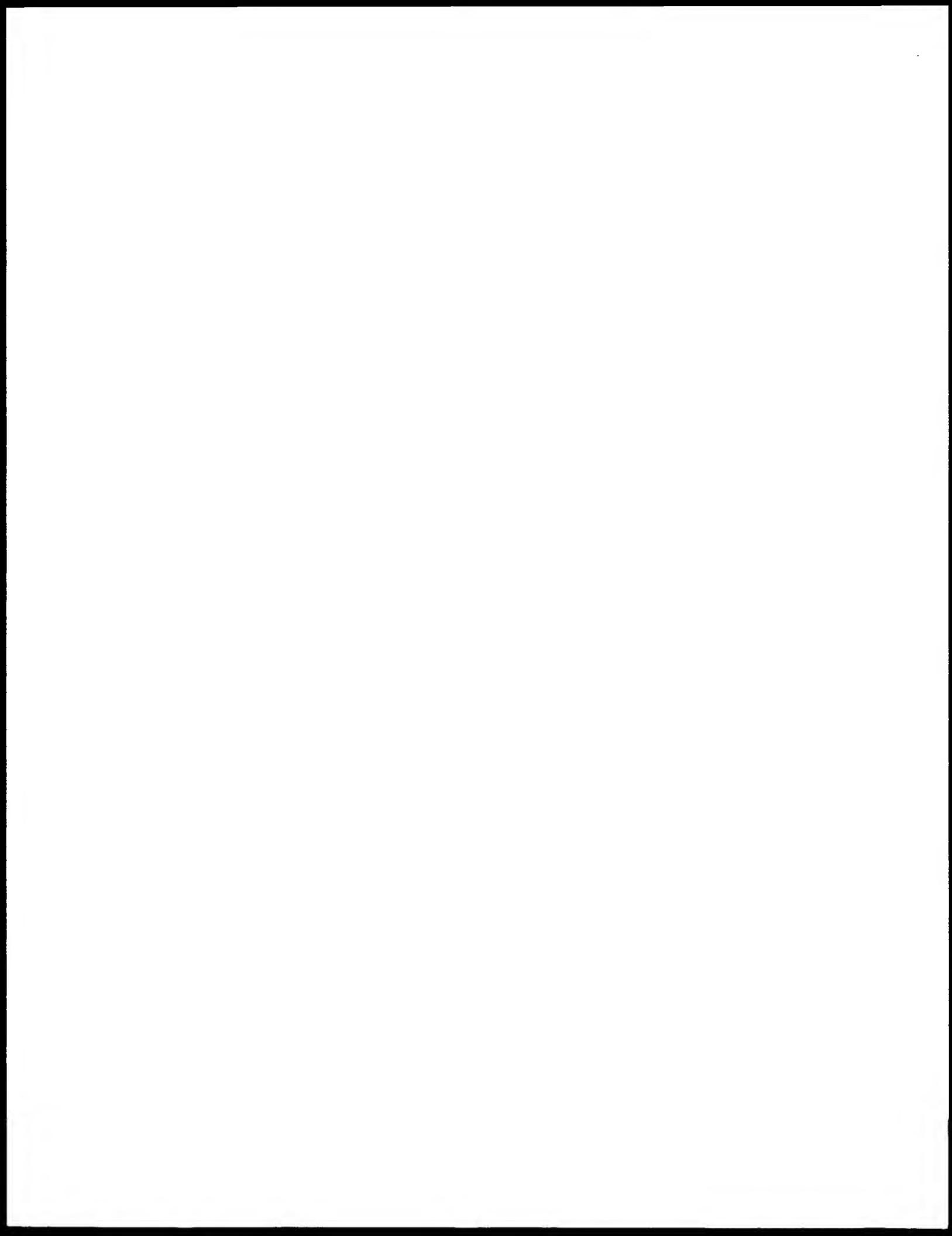
15 Prior to the disclosure of the present invention herein, the standard methodology for the generation of a primary culture of ORS keratinocytes consisted of the plucking of an anagen (i.e., growing hair shaft) hair followed by a careful microscopic dissection to remove the hair bulbs and the infundibular hair shaft. The resulting outer root sheath was then placed on the culture insert for initiation of the primary keratinocyte culture. However, numerous 20 subsequent studies (approximately 200), wherein the anagen hair was placed directly on the culture insert without performing the initial micro-dissection to remove the hair bulbs and the infundibular hair shaft, have demonstrated that such tedious and time-consuming dissection of the plucked anagen hair was not required. This has served to markedly simplify the handling process, reduce the risk for contamination, and resulted in more efficient initiation of 25 keratinocyte cell plating.

Accordingly, it is an object of the present invention to provide improved and simplified methods for the generation of keratinocytes or keratinocyte precursors from outer root sheath cells (ORS cells) in fully defined culture conditions for the treatment of various types of skin defects (e.g., chronic wounds such as leg ulcers, diabetic

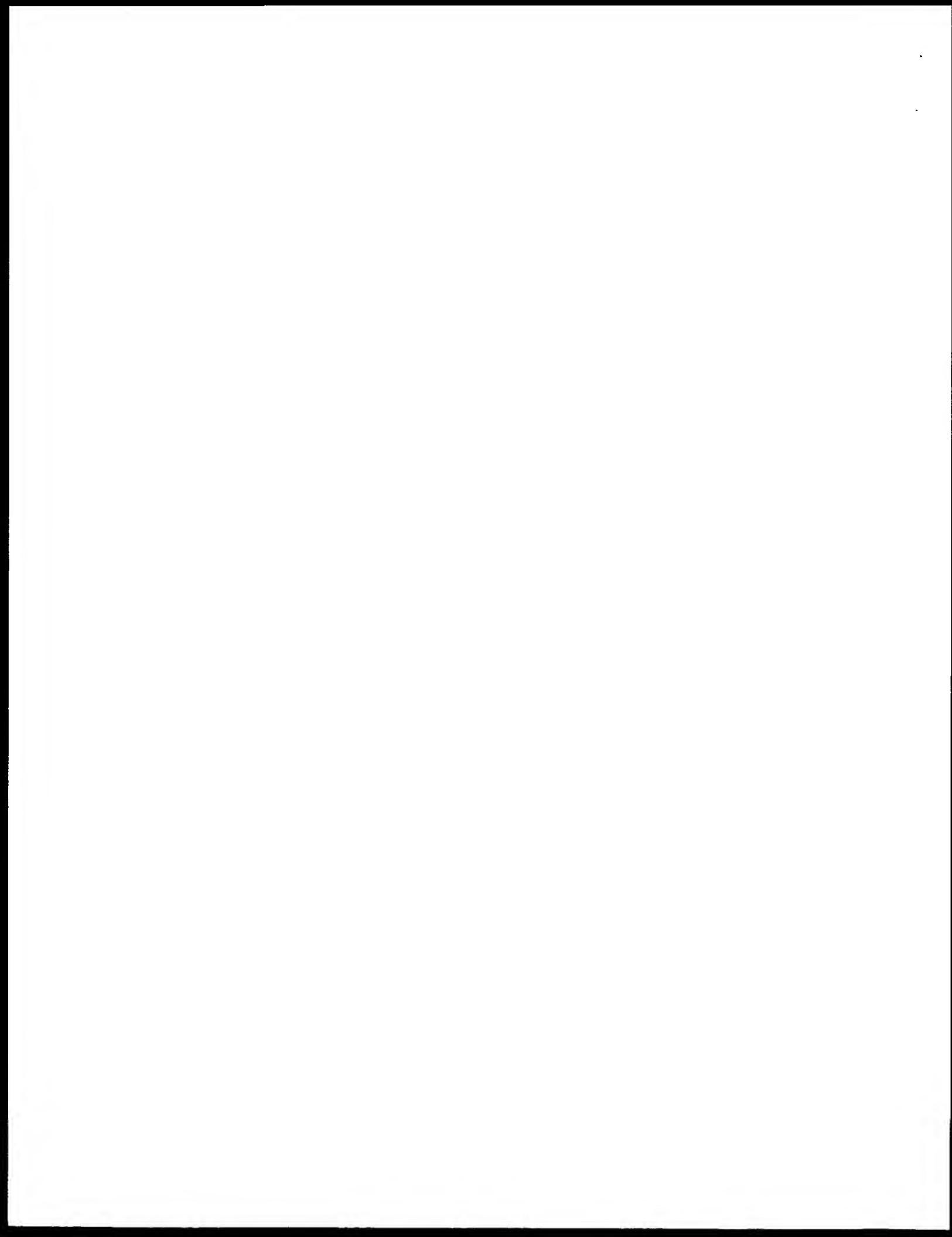


## CLAIMS

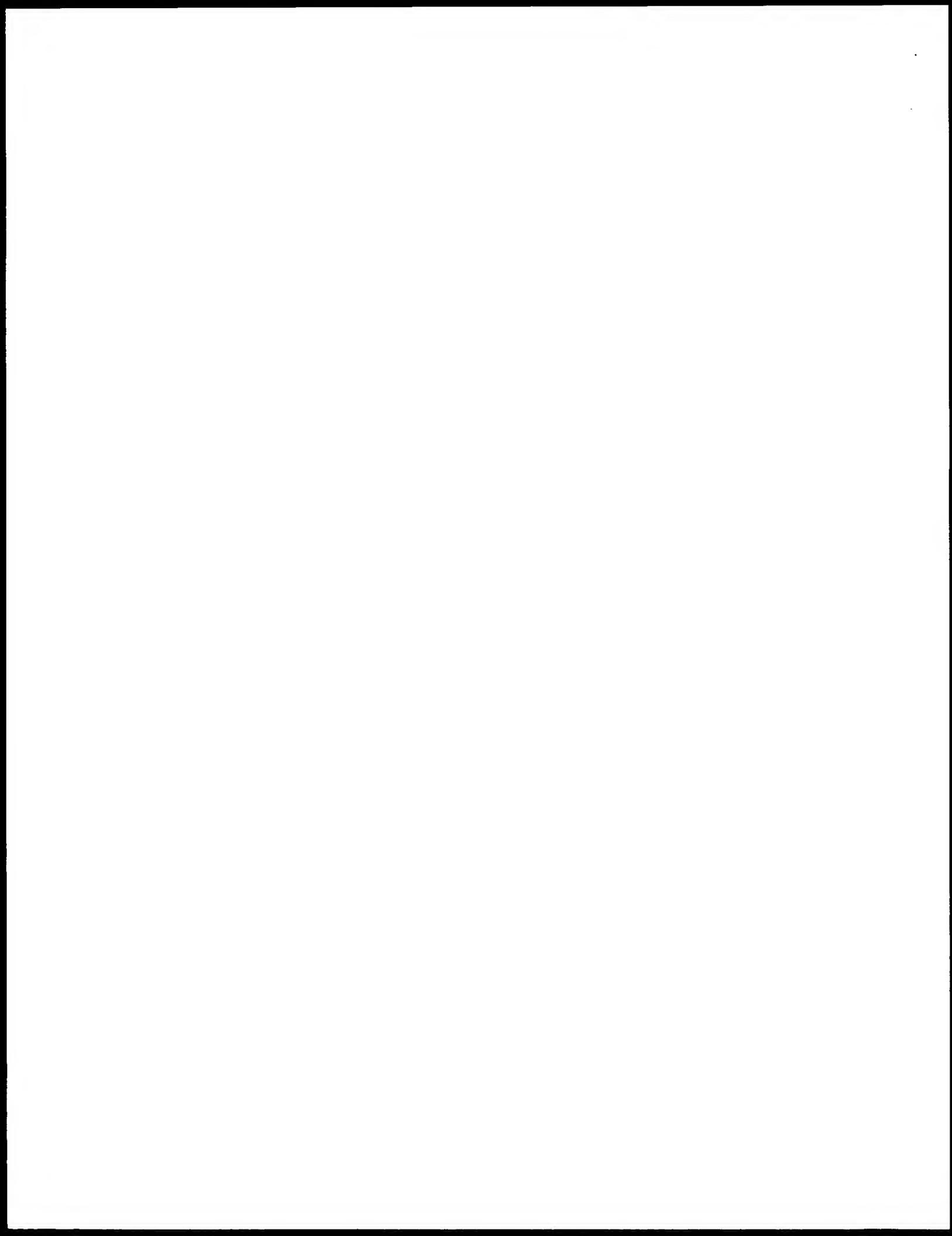
1. A method for the treatment of a skin defect comprising
  - (a) culturing an intact hair follicle of an anagenic hair to obtain outer root sheath cells;
  - (b) culturing said outer root sheath cells to obtain keratinocyte precursor cells;
  - (c) preparing an epidermal or dermal equivalent comprising said keratinocyte precursor cells; and
  - (d) applying a portion of said epidermal or dermal equivalent to said defect.
2. The method of claim 1, wherein said outer root sheath cells are autologous cells obtained from an individual who will subsequently undergo treatment for a skin defect.
3. The method of claim 1, wherein said outer root sheath cells are homologous cells.
4. The method of claim 1, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing only homologous or autologous biological supplements.
5. The method of claim 1, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing human serum in a concentration of less than 5%.
6. The method of claim 1, wherein the culture density of said keratinocyte precursor cells is between about  $3 \times 10^4$  cells/cm<sup>2</sup> and about  $1 \times 10^5$  cells/cm<sup>2</sup>.
7. The method of claim 1, wherein said epidermal or skin equivalents are coated on their top or cornified side with a fibrin glue.



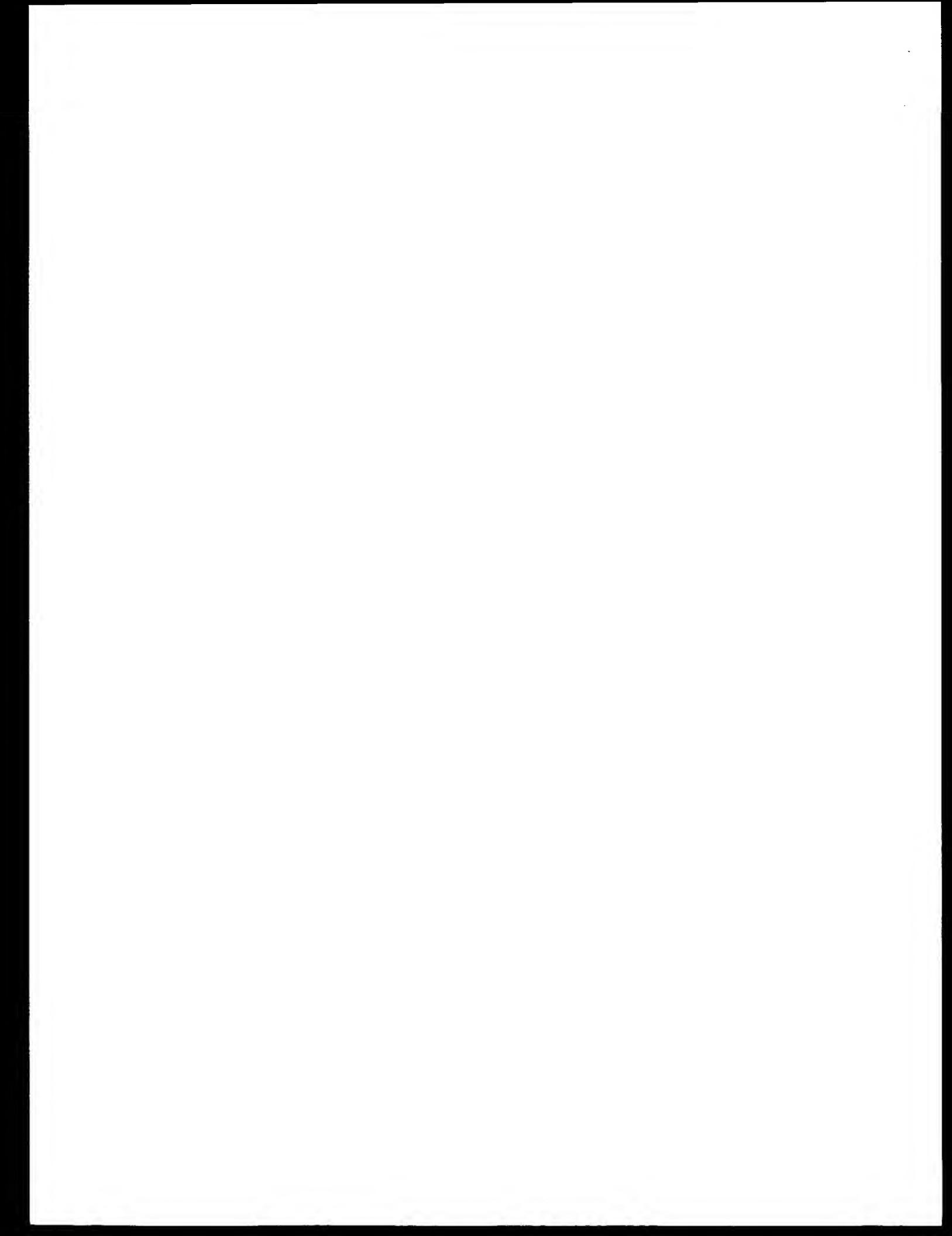
8. The method of claim 1, wherein said epidermal or skin equivalents are coated on their top or cornified side with a carrier membrane.
9. The method of claim 1, wherein the graft thickness for said epidermal equivalents is 50-150 microns.
10. The method of claim 1, wherein the graft thickness for said complex skin equivalents does not exceed 0.4 mm.
11. A method for the treatment of a skin defect comprising
  - (a) culturing an intact hair follicle of an anagenic hair to obtain outer root sheath cells;
  - (b) culturing said outer root sheath cells to obtain keratinocyte precursor cells;
  - (c) preparing an epidermal or dermal equivalent comprising said keratinocyte precursor cells; and
  - (d) applying a portion of said epidermal or dermal equivalent to said defect wherein all culturing of cells is performed in a medium which utilizes autologous or homologous human serum in a concentration of preferably less than approximately 5%.
12. The method of claim 11, wherein said anagen or growing hair is cultured *in toto*.
13. The method of claim 11, wherein said keratinocyte precursor cells have a culture density of between approximately  $3 \times 10^4$  cells/cm<sup>2</sup> to approximately  $1 \times 10^5$  cells/cm<sup>2</sup>.
14. The method of claim 11, wherein said outer root sheath cells are homologous cells.
15. The method of claim 11, wherein the culture density of said keratinocyte precursor cells is between about  $3 \times 10^4$  cells/cm<sup>2</sup> and about  $1 \times 10^5$  cells/cm<sup>2</sup>.



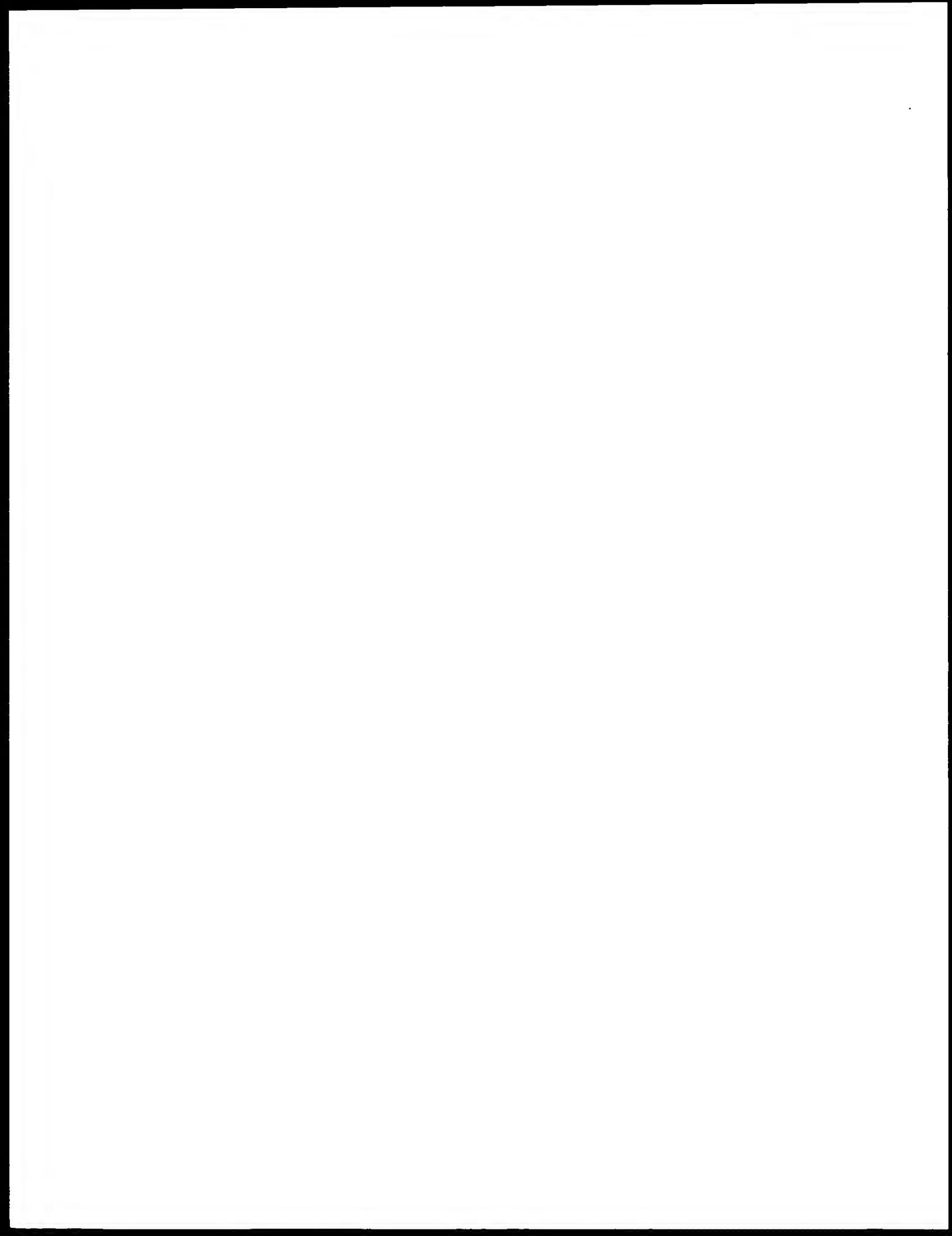
16. The method of claim 11, wherein said epidermal or skin equivalents are coated on their top or cornified side with a fibrin glue.
17. The method of claim 11, wherein said epidermal or skin equivalents are coated on their top or cornified side with a carrier membrane.
18. The method of claim 11, wherein the graft thickness for said epidermal equivalents is 50-150 microns.
19. The method of claim 11, wherein the graft thickness for said complex skin equivalents does not exceed 0.4 mm.
20. A method for the treatment of a skin defect comprising
  - (a) culturing an intact hair follicle of an anagenic hair to obtain outer root sheath cells;
  - (b) culturing said outer root sheath cells to obtain keratinocyte precursor cells;
  - (c) preparing an epidermal or dermal equivalent comprising said keratinocyte precursor cells; and
  - (d) applying a portion of said epidermal or dermal equivalent to said defect wherein said epidermal or dermal equivalent is coated on its top or cornified side with a fibrin glue.
21. The method of claim 20, wherein said anagen or growing hair is cultured *in toto*.
22. The method of claim 20, wherein said outer root sheath cells are autologous cells obtained from an individual who will subsequently undergo treatment for a skin defect.
23. The method of claim 20, wherein said outer root sheath cells are homologous cells.



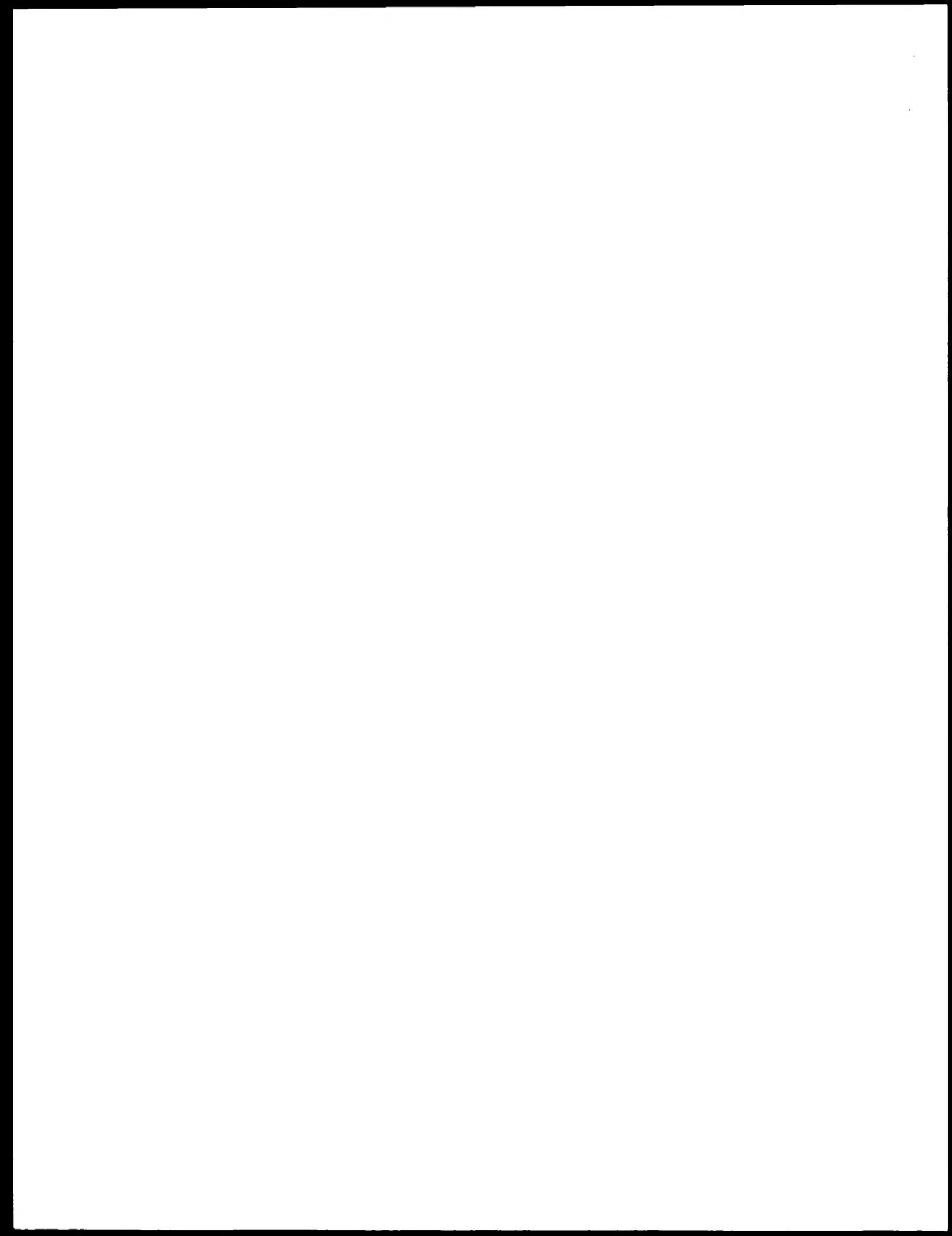
24. The method of claim 20, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing only homologous or autologous biological supplements.
25. The method of claim 20, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing human serum in a concentration of less than 5%.
26. The method of claim 20, wherein the culture density of said keratinocyte precursor cells is between about  $3 \times 10^4$  cells/cm<sup>2</sup> and about  $1 \times 10^5$  cells/cm<sup>2</sup>.
27. The method of claim 20, wherein said epidermal or skin equivalents are coated on their top or cornified side with a fibrin glue which contains one or more anti-microbial, anti-fungal, or anti-viral agents emulsified therein.
28. A method for the selection of keratinocyte precursor cells from the outer root sheath of hair for subsequent use in a composition for healing a skin defect, comprising the steps of:
- plucking of an intact anagen hair;
  - primary-culturing the outer root sheath-derived keratinocyte precursor cells by adhering said intact anagen hair to a microporous membrane, which possesses growth-arrested/limited feeder cells on its undersurface so as to select for keratinocyte precursor cells from the outer root sheath of hair;
  - organotypically-culturing the outer root sheath cells harvested from said primary cultures by inoculating a microporous membrane which also possesses growth-arrested/limited feeder cells on its undersurface;
  - generating an epidermal or complex skin equivalent, for subsequent use as a graft insert, by placing a carrier membrane on top of said organotypic-culture from step (c) and detaching said complex skin or epidermal equivalent, which is comprised



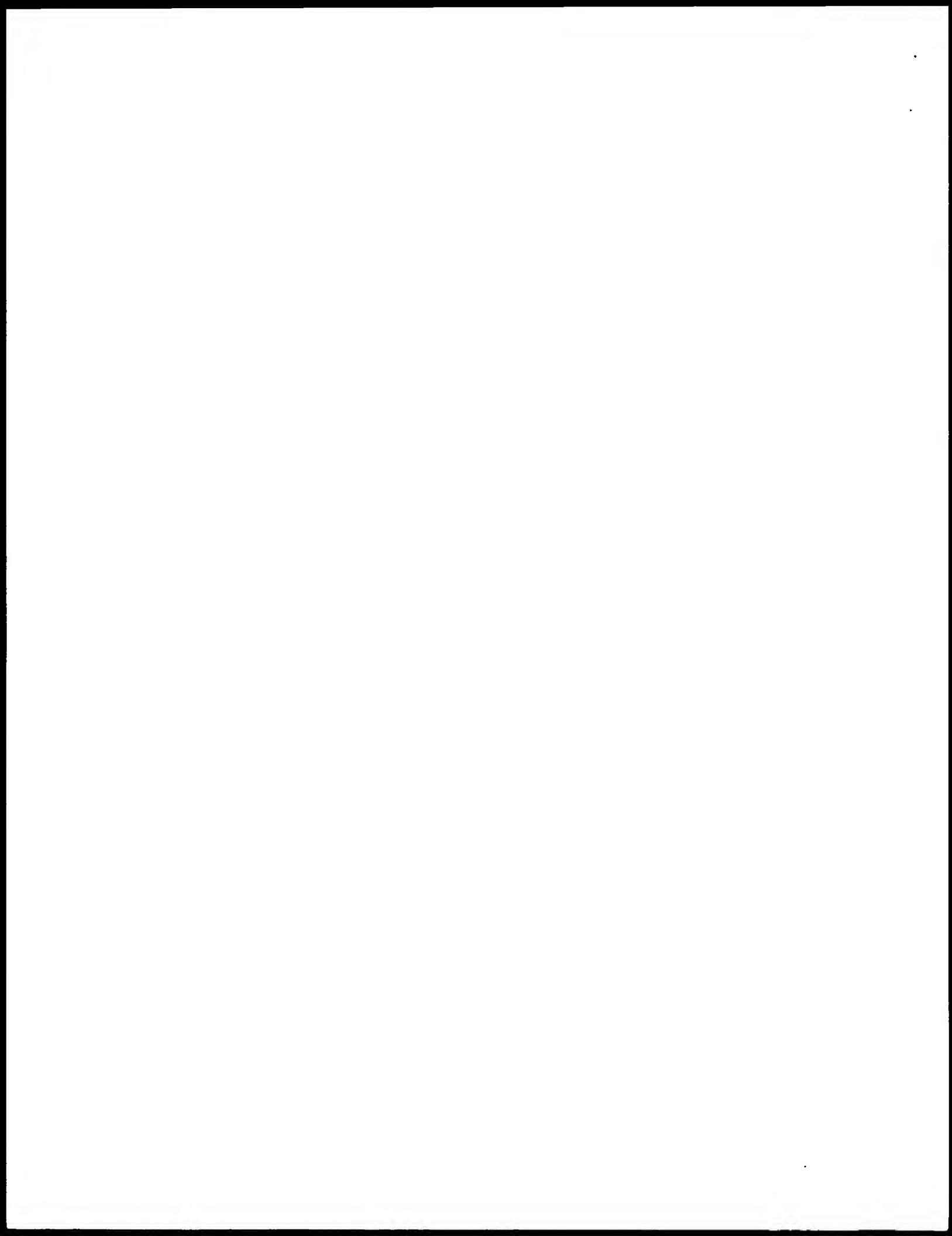
- of the keratinocyte precursor cells and carrier membrane, together as a single, laminar unit;
- (e) contacting said epidermal or skin equivalent with a skin defect present on an individual, and immobilizing said epidermal or skin equivalent at the site of contact.
29. The method of claim 28, wherein said outer root sheath cells are autologous cells derived from the individual who will subsequently undergo treatment for a skin defect.
30. The method of claim 28, wherein said outer root sheath cells are homologous cells.
31. The method of claim 28, wherein the culture density of said keratinocyte precursor cells is between about  $3 \times 10^4$  cells/cm<sup>2</sup> and about  $1 \times 10^5$  cells/cm<sup>2</sup>.
32. The method of claim 28, wherein the culture density of said growth-arrested/limited feeder cells on said microporous membrane is between about  $1 \times 10^4$  cells/cm<sup>2</sup> and about  $5 \times 10^4$  cells/cm<sup>2</sup>.
33. The method of claim 28, wherein said growth-arrested/limited feeder cells are banked or immortalized cells.
34. The method of claim 28, wherein said primary and organotypic cultures utilize autologous or homologous human serum.
35. The method of claims 28, wherein said primary and organotypic cultures utilize autologous or homologous human serum in a concentration of less than about 5%.
36. The method of claim 28, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing only homologous or autologous biological supplements.



37. The method of claim 28, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing only homologous or autologous releasates from blood components.
38. The method of claims 28, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing only homologous or autologous releasates from blood components at a concentration of about 0.1% to about 20%.
39. The method of claim 28, wherein said epidermal equivalents are coated on their top or cornified side with a fibrin glue.
40. The method of claims 28, wherein said epidermal equivalents are coated on their top or cornified side with a carrier membrane.
41. The method of claims 28, wherein the graft thickness for said epidermal equivalents is 50-150 microns.
42. The method of claims 28, wherein the graft thickness for said complex skin equivalents does not exceed 0.4 mm.
43. The method of claim 28, wherein said microporous membrane is coated by one or ore extracellular matrix substances selected from a group consisting of: fibrin, fibronectin, collagens, laminins and hyaluronan.
44. The method of claims 28, wherein said microporous membrane possesses a growth-arrested/limited feeder cell system on its undersurface with said feeder cells of at least one type of cell selected from the group comprising human dermal fibroblasts, epidermal cells, mesenchymal cells, neuronal cells and endothelial cells.



45. The method of claim 28, wherein said carrier membrane is made from one or more types of materials selected from the group comprising polyester, PTFE, polyurethane, hyaluronic acid, polylactic acid, collagen, or a silicone or vaseline gauze dressing.
46. The method of claim 28, wherein the size of said epidermal equivalent is selected from the group consisting of 1.0 cm, 1.5 cm, 2.0 cm, and 2.5 cm in diameter.
47. A method of shipping or transporting epidermal equivalents comprising:
  - (a) detaching said epidermal equivalents from a culture medium, and
  - (b) transferring said epidermal equivalents onto a transport medium.
48. The method of claim 47, wherein said epidermal equivalents are coated on their top or cornified side with a carrier membrane.
49. The method of claim 48, wherein said epidermal equivalents are further sealed and shipped for future use in grafting.
50. The method of claim 47, wherein said transport medium comprises a solidified or gelled medium.
51. The method of claim 50, wherein said solidified or gelled medium is selected from the group consisting of agarose, methyl cellulose, or another gelifying substance.



## PATENT COOPERATION TREATY

PCT

REC'D 25 OCT 2001

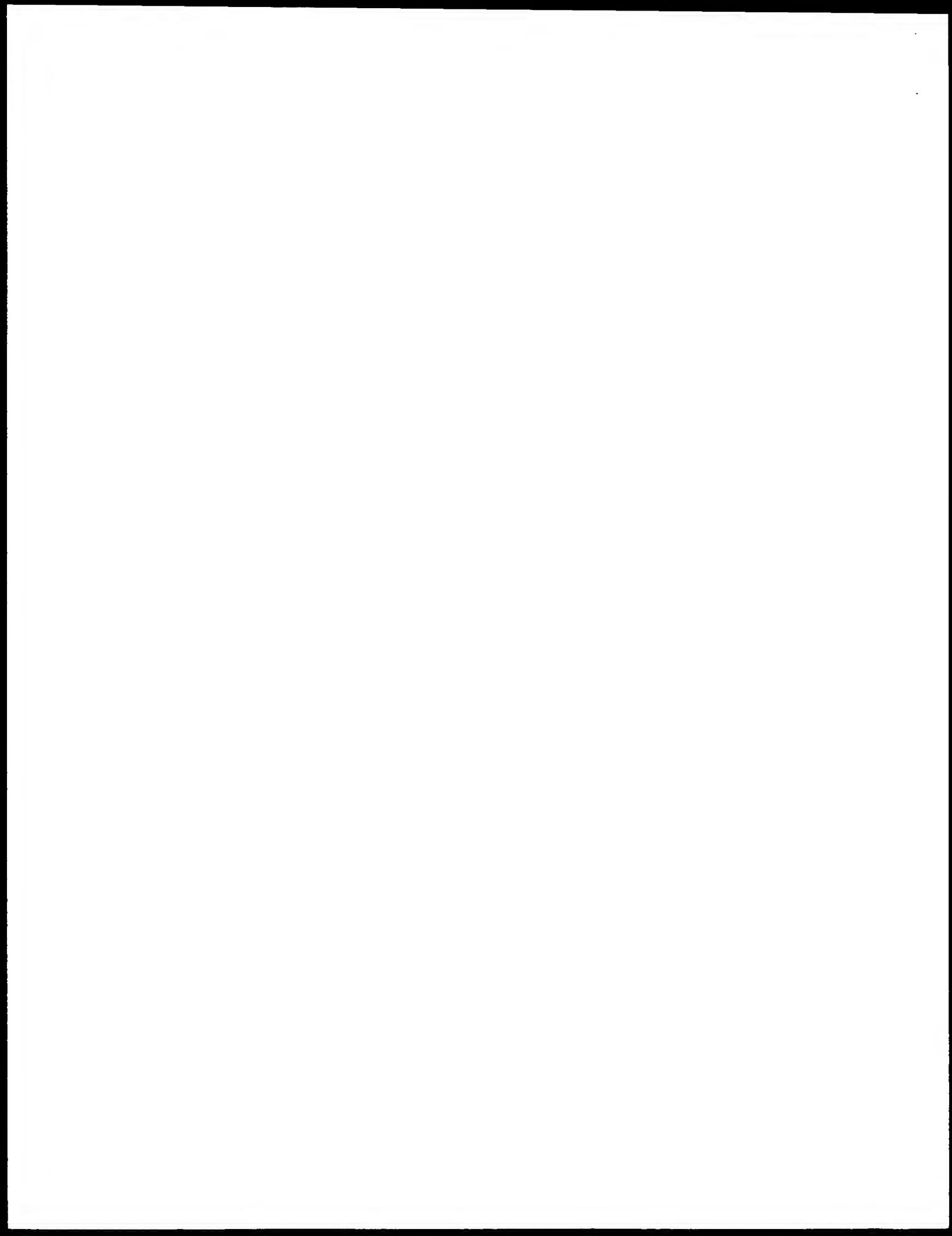
## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference GWS/MRM/22559	<b>FOR FURTHER ACTION</b>		See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/IB00/01076	International filing date (day/month/year) 20/07/2000	Priority date (day/month/year) 20/07/1999	
International Patent Classification (IPC) or national classification and IPC C12N5/00			
Applicant EPITECH S.A. et al.			
<p>1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of 10 sheets, including this cover sheet.</p> <p><input checked="" type="checkbox"/> This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).</p> <p>These annexes consist of a total of 9 sheets.</p>			
<p>3. This report contains indications relating to the following items:</p> <ul style="list-style-type: none"> <li>I <input checked="" type="checkbox"/> Basis of the report</li> <li>II <input checked="" type="checkbox"/> Priority</li> <li>III <input checked="" type="checkbox"/> Non-establishment of opinion with regard to novelty, inventive step and industrial applicability</li> <li>IV <input checked="" type="checkbox"/> Lack of unity of invention</li> <li>V <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement</li> <li>VI <input type="checkbox"/> Certain documents cited</li> <li>VII <input type="checkbox"/> Certain defects in the international application</li> <li>VIII <input type="checkbox"/> Certain observations on the international application</li> </ul>			

Date of submission of the demand 19/02/2001	Date of completion of this report 23.10.2001
Name and mailing address of the international preliminary examining authority:   European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer  Nichogiannopoulou, A Telephone No. +49 89 2399 8054





**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/IB00/01076

**I. Basis of the report**

1. With regard to the **elements** of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):

**Description, pages:**

1,2,4-26 as originally filed

3,3a as received on 26/09/2001 with letter of 25/09/2001

**Claims, No.:**

1-51 as received on 26/09/2001 with letter of 25/09/2001

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

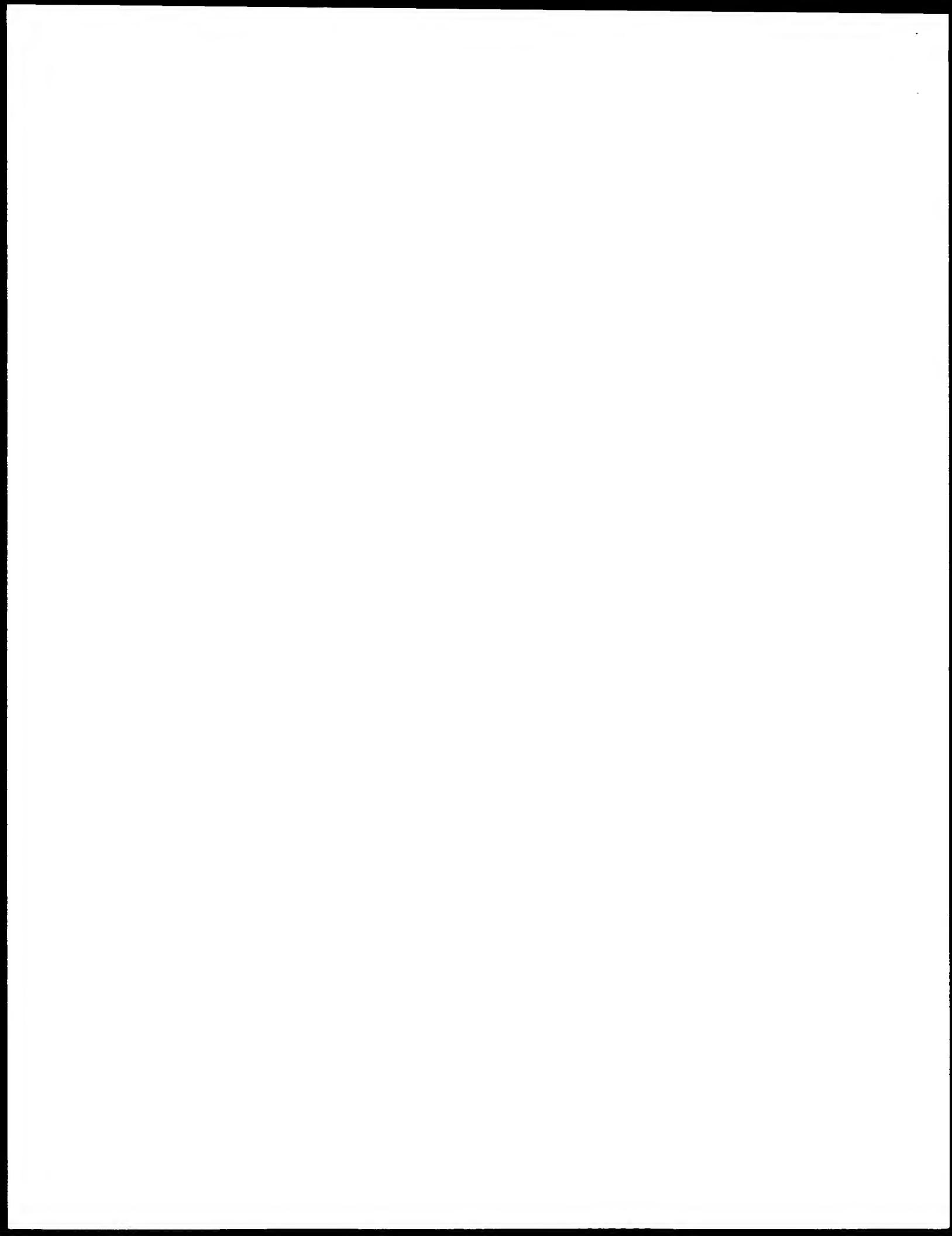
- the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- the language of publication of the international application (under Rule 48.3(b)).
- the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- contained in the international application in written form.
- filed together with the international application in computer readable form.
- furnished subsequently to this Authority in written form.
- furnished subsequently to this Authority in computer readable form.
- The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- the description, pages:
- the claims, Nos.:
- the drawings, sheets:



# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IB00/01076

5.  This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

*(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)*

6. Additional observations, if necessary:

## II. Priority

1.  This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

- copy of the earlier application whose priority has been claimed.
- translation of the earlier application whose priority has been claimed.

2.  This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

**see separate sheet**

## III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

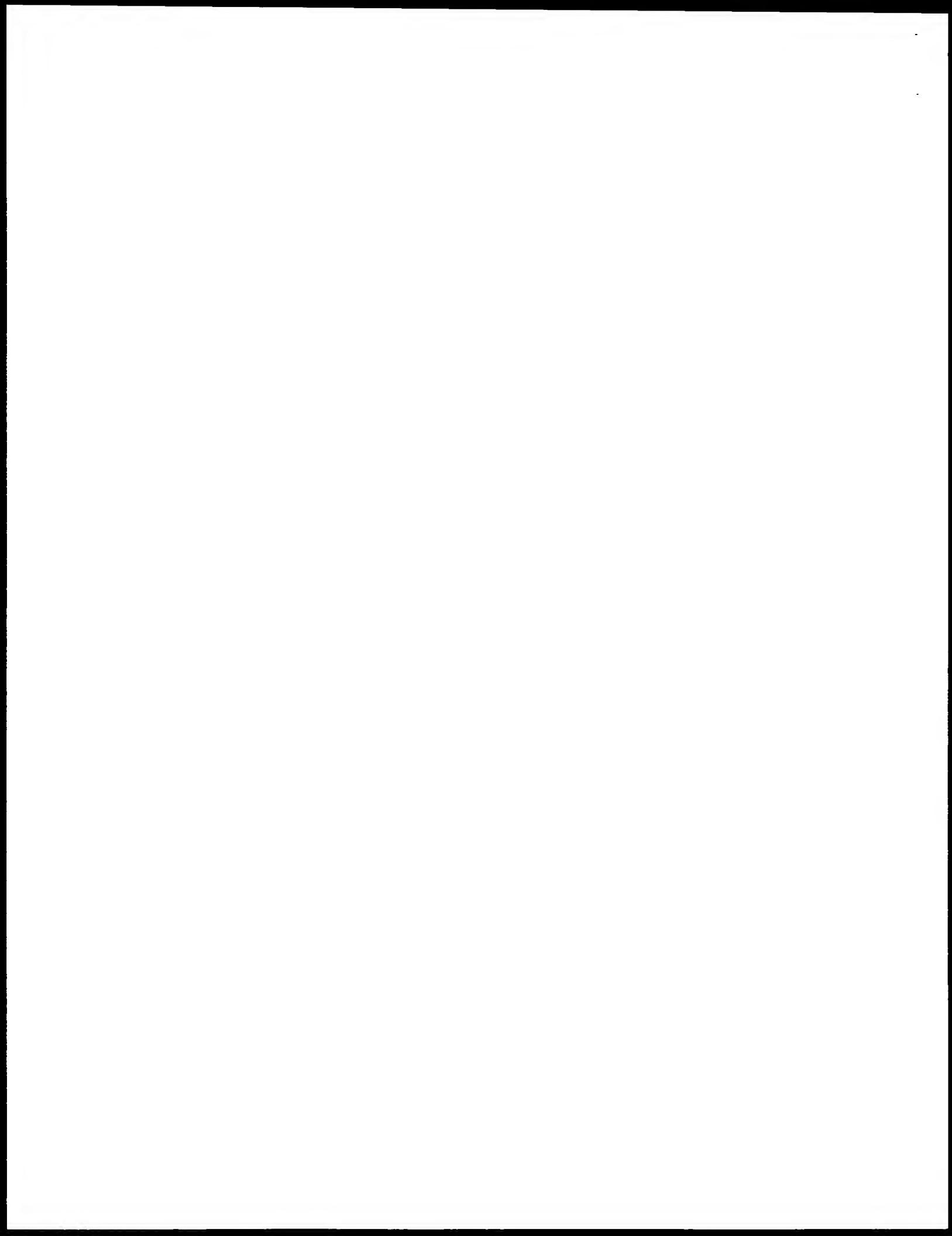
1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

- the entire international application.

- claims Nos. 1-46.

because:

- the said international application, or the said claims Nos. 1-46 relate to the following subject matter which does not require an international preliminary examination (*specify*):  
**see separate sheet**
- the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):
- the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.
- no international search report has been established for the said claims Nos. .



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/IB00/01076

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

- the written form has not been furnished or does not comply with the standard.
- the computer readable form has not been furnished or does not comply with the standard.

**IV. Lack of unity of invention**

1. In response to the invitation to restrict or pay additional fees the applicant has:

- restricted the claims.
- paid additional fees.
- paid additional fees under protest.
- neither restricted nor paid additional fees.

2.  This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

- complied with.
- not complied with for the following reasons:

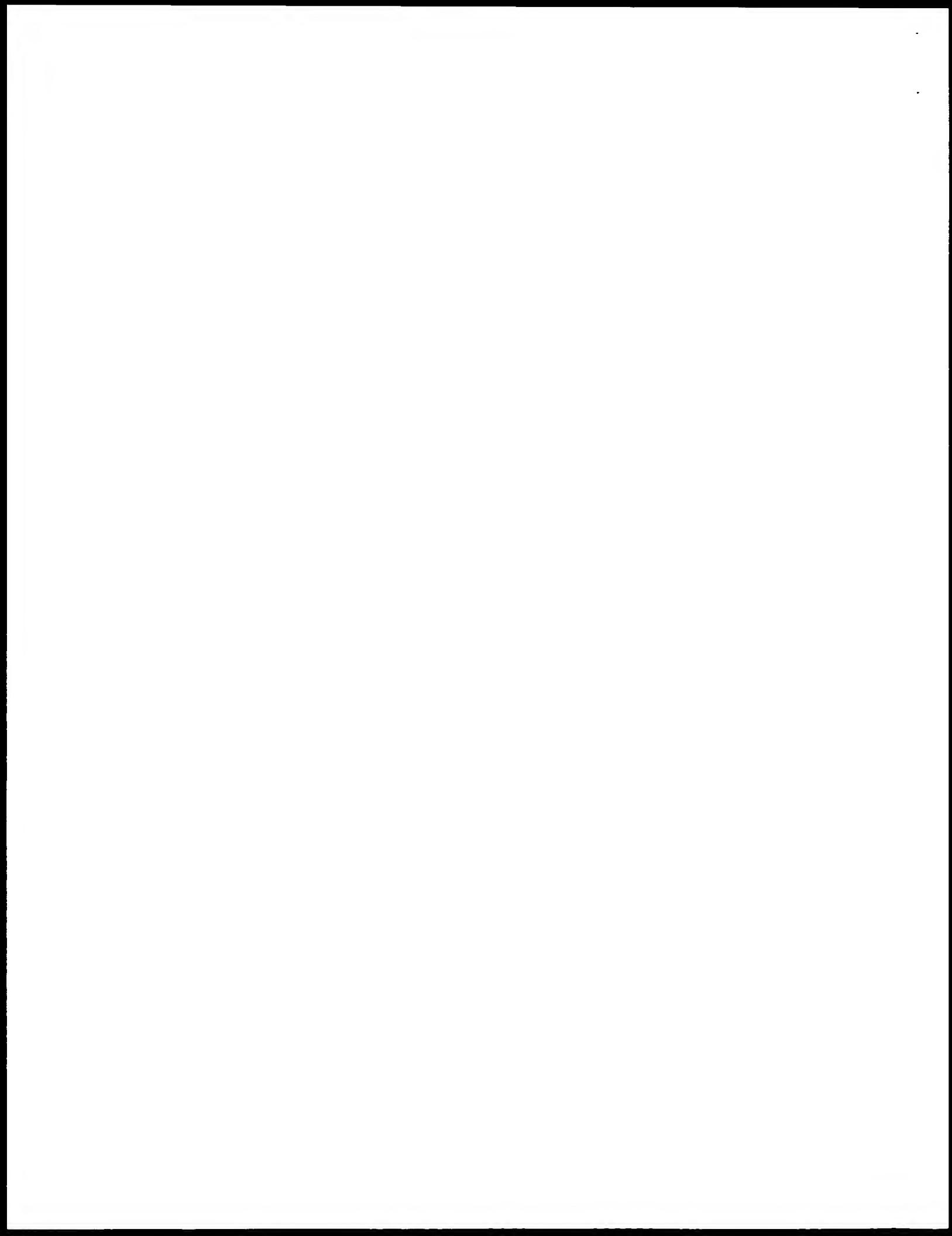
4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

- all parts.
- the parts relating to claims Nos. 1-46.

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

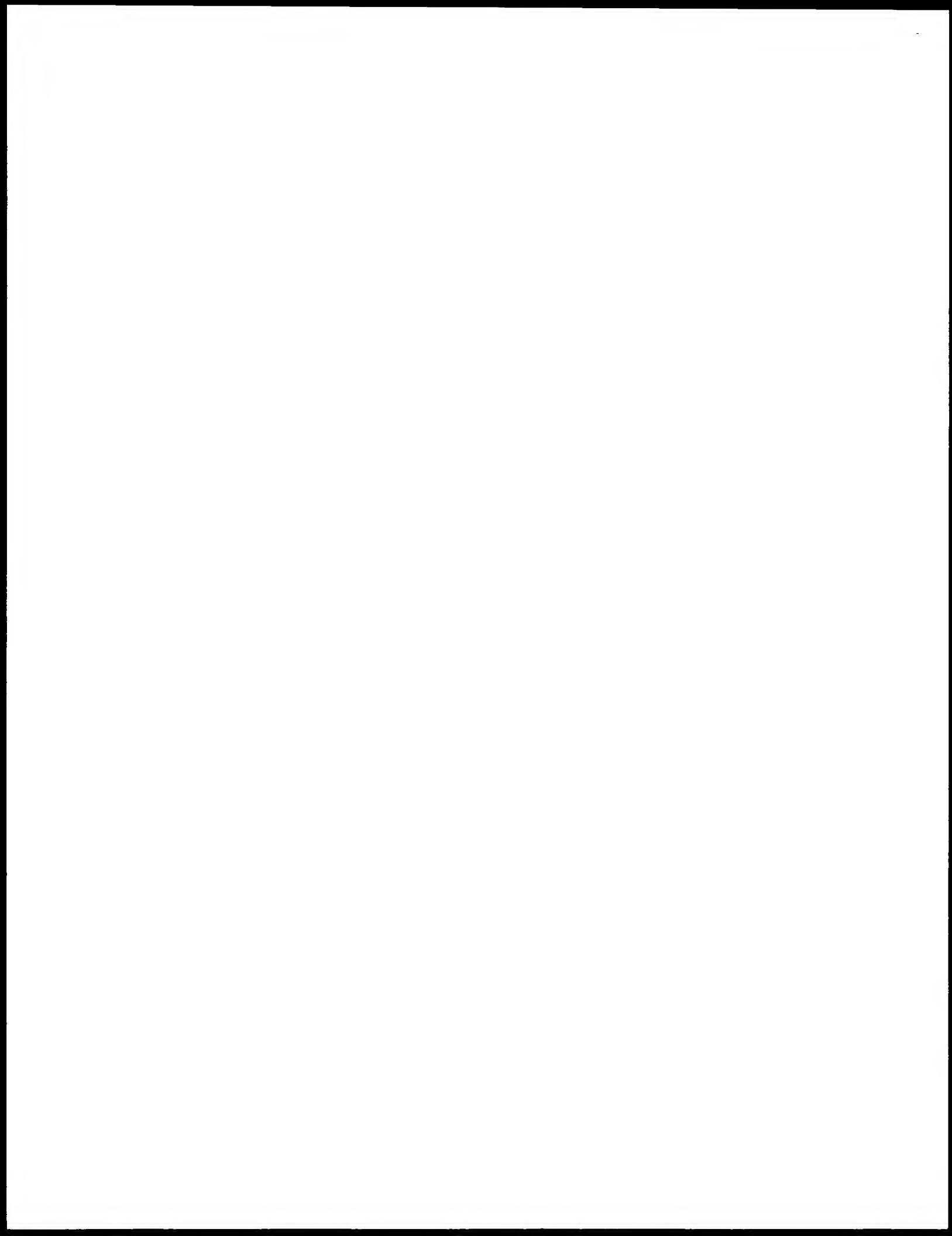
Novelty (N)	Yes:	Claims 1-46
	No:	Claims
Inventive step (IS)	Yes:	Claims 8, 13-27, 39-46
	No:	Claims 1-7, 9-12, 28-38
Industrial applicability (IA)	Yes:	Claims see box III
	No:	Claims



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/IB00/01076

2. Citations and explanations  
**see separate sheet**



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

---

International application No. PCT/IB00/01076

**Re Item I**

**Basis of the report**

1. The amendments filed with the letter of 25.09.2001 are formally allowable under Article 34(2)(b) PCT because they do not introduce subject-matter extending beyond the content of the application as filed.

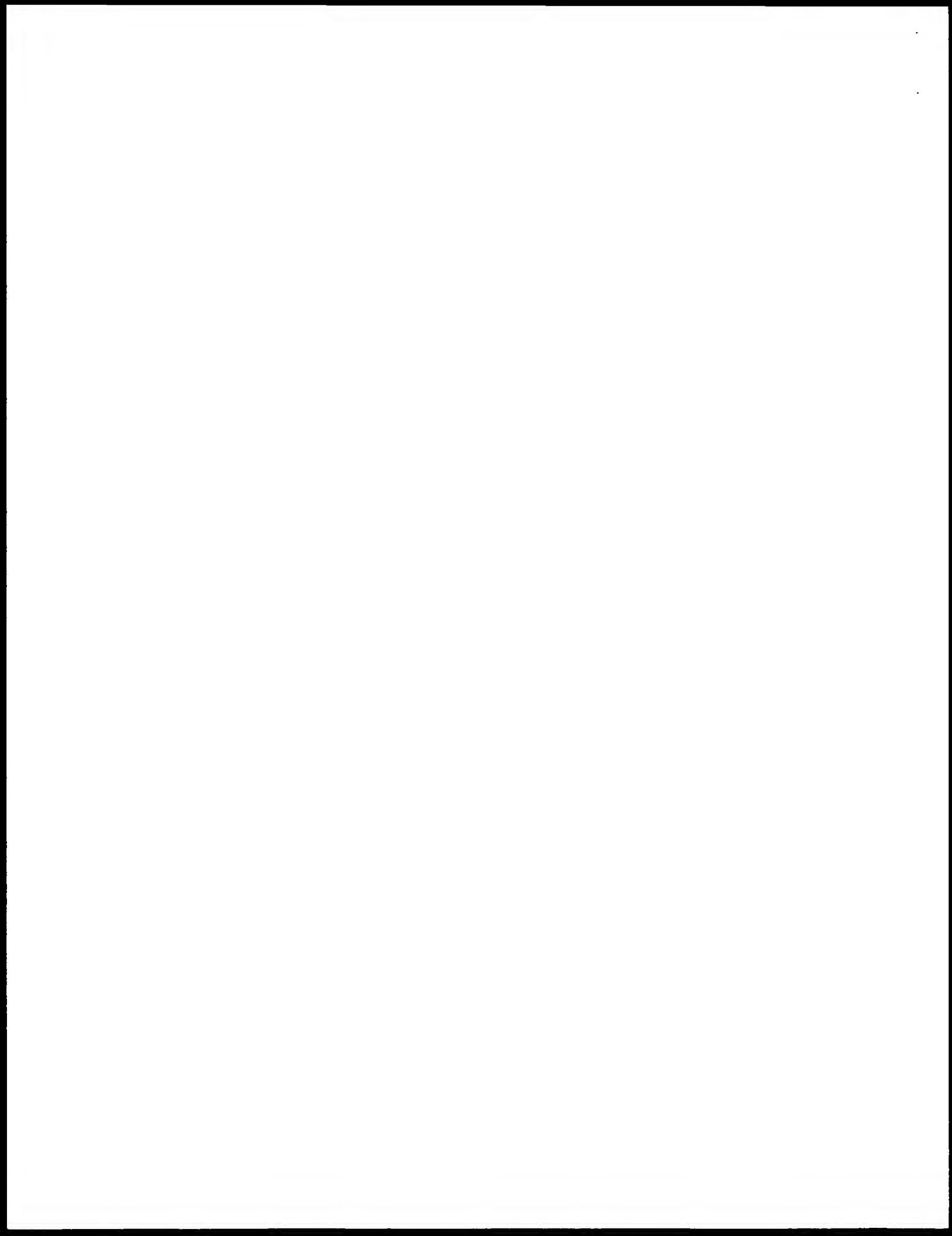
**Re Item II**

**Priority**

1. The following document was published prior to the international filing date but later than the priority date claimed (P-document):

P1: US-A-5 968 546 (BAUR MARCUS ET AL) 19 October 1999 (1999-10-19)

2. The priority document pertaining to the present application was not available at the time of establishing this report. Hence, the current assessment is based on the assumption that all claims enjoy priority rights from the filing date of the priority document (20.07.1999). If it later turns out that this assumption is incorrect, P1 will become relevant to the assessment of whether the present application satisfies the criteria set forth in Article 33(2) and (3) PCT.



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/IB00/01076

**Re Item III**

**Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

1. No meaningful examination could be performed for claims 1-46 for the following reason:

Claims 1-46 -as far as they concern *in vivo* methods- relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

**Re Item IV**

**Lack of unity of invention**

1. The present application addresses the generation of *in vitro* cultured epidermal equivalents and methods for their transport.

The generation of epidermal equivalents, in particular from outer root sheath cells, is widely known from the prior art (see Limat et al., 1996).

In the light of the prior art, the problems and corresponding solutions of the present application can be summarized as follows:

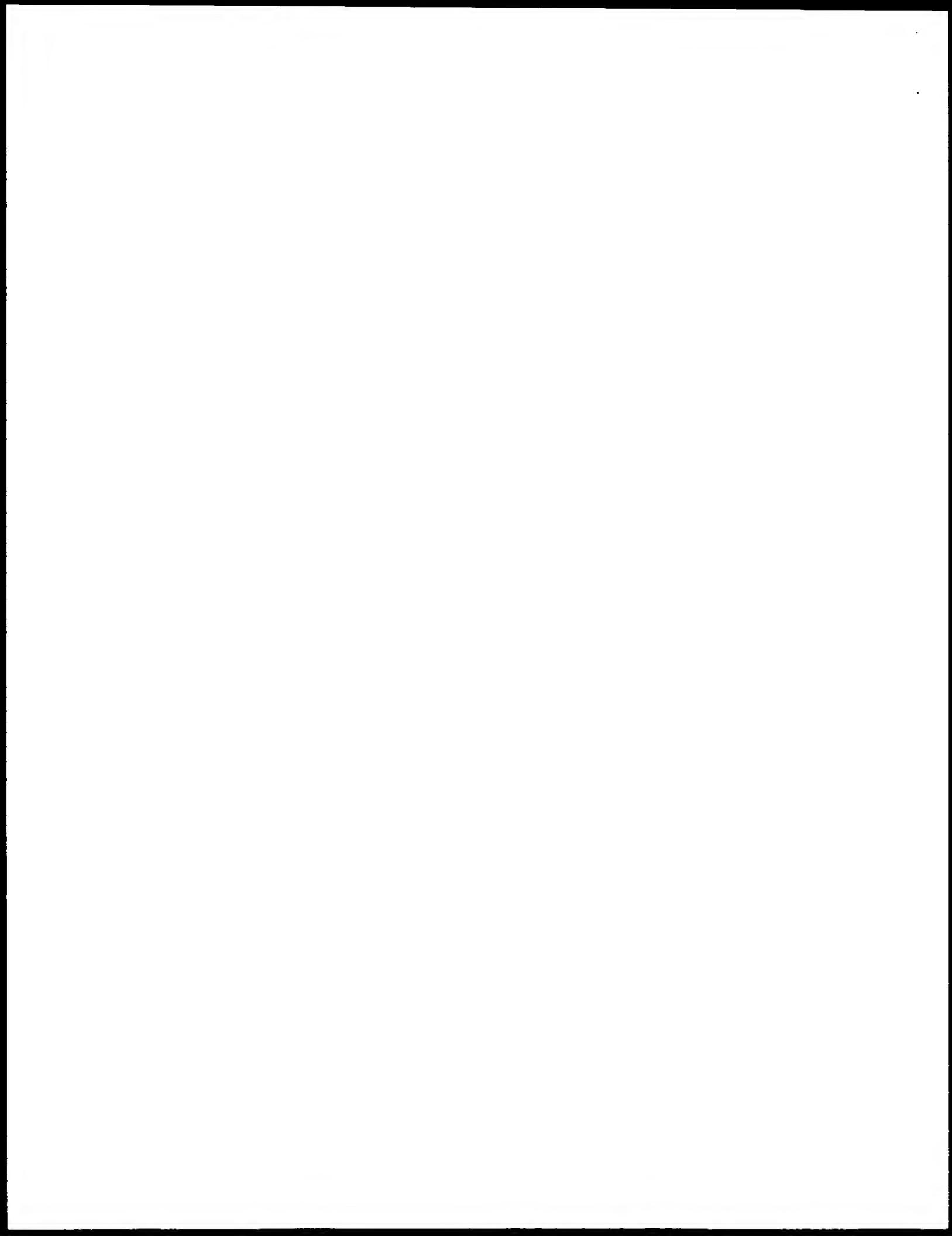
**Problem I:** Providing alternatives for the generation of epidermal equivalents from outer root sheath cells.

**Solution 1:** claims 1-3, 6, 9, 10 all completely and 4, 5, 7, 8, 12, 21, 28-46 all partially

A method for the generation of an epidermal equivalent from keratinocyte precursors from outer root sheath (ORS) cells by culture of anagen hair *in toto*.

**Solution 2:** claims 11, 13-15, 18, 19 all completely and 4, 5, 12, 16, 17, 24, 25, 34-38 all partially

A method for the generation of an epidermal equivalent from keratinocyte precursors from ORS cells by culturing in media



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

---

International application No. PCT/IB00/01076

supplemented with less than 5% autologous or homologous human serum.

Solution 3: claims 20, 22, 23, 26, 27 all completely and 7, 8, 16, 17, 21, 24, 25, 28-46 all partially

A method for the generation of an epidermal equivalent from keratinocyte precursors from ORS cells wherein said epidermal equivalent is coated on its top side with a fibrin glue.

Problem II: Providing a method for the transport of cultured epidermal equivalents.

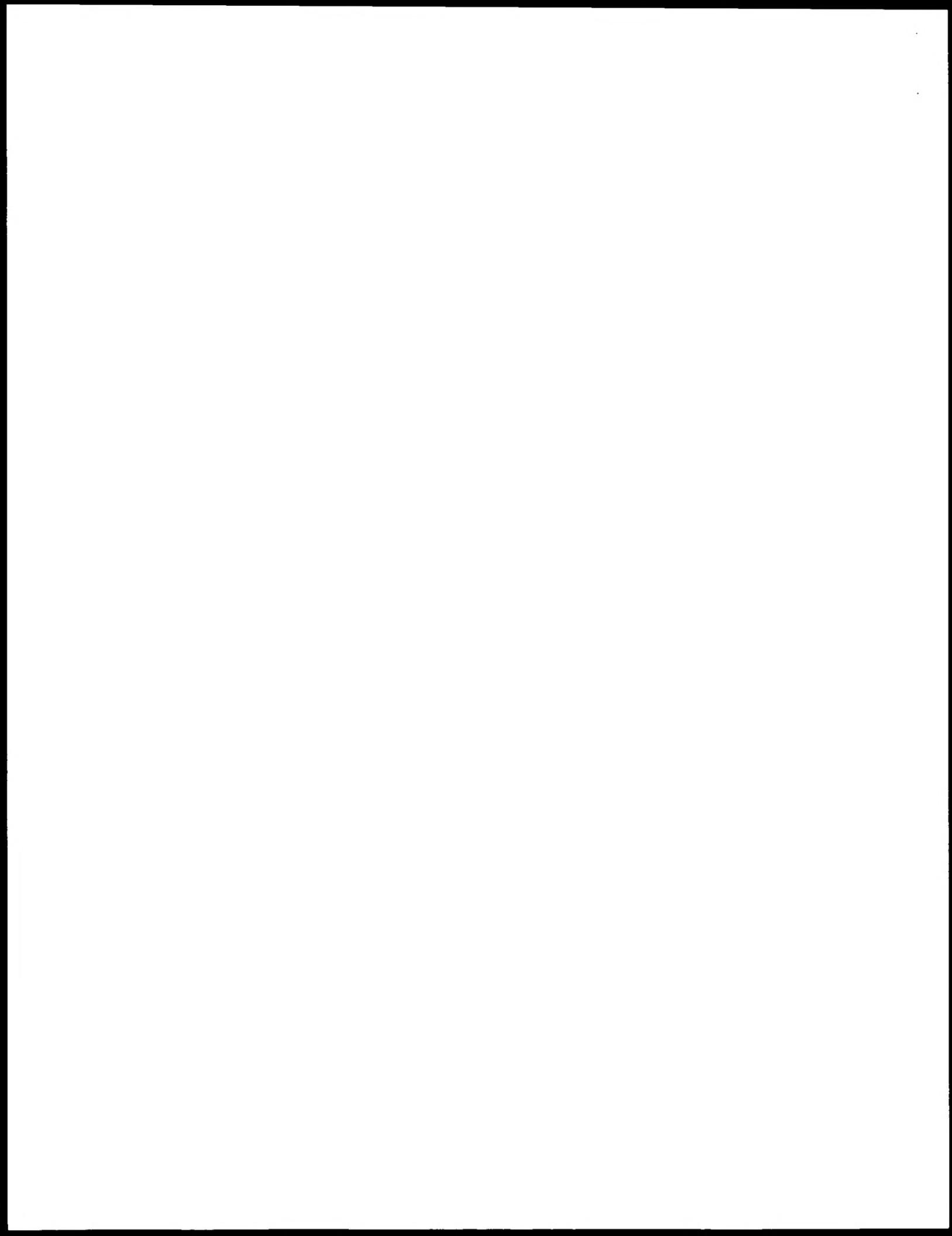
Solution 4: claims 47-51

A method of detaching epidermal equivalents and transferring them into an appropriate medium

Solutions 1-3 of problem I and solution 4 of problem II represent *a priori* distinct problems and their respective solutions as brought forward in the present application are not so linked as to form a single inventive concept. Since the special technical features outlined above are not the same or corresponding, the requirement of unity in the sense of Rule 13.2 PCT is not fulfilled. Consequently there is lack of unity, and there are four different inventions not belonging to a common inventive concept.

**Re Item V**

**Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/IB00/01076

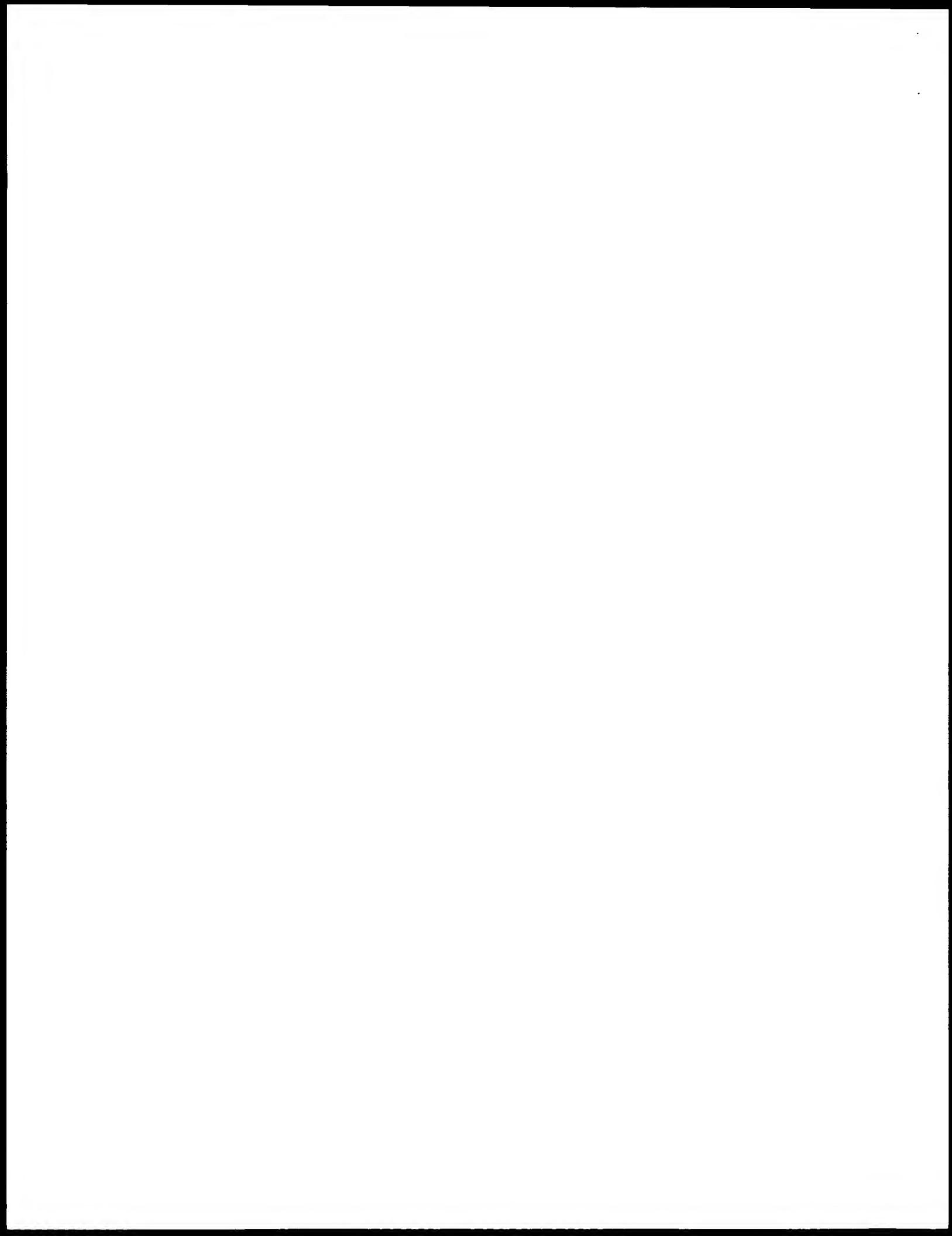
1. Reference is made to the following documents:

- D1: DE 196 51 992 A (TOLOCZYKI CHRISTIAN DR) 25 June 1998 (1998-06-25)
- D2: LENOIR-VIALE M -C ET AL: "Epidermis reconstructed from the outer root sheath of human hair follicle: Effect of retinoic acid." ARCHIVES OF DERMATOLOGICAL RESEARCH, vol. 285, no. 4, 1993, pages 197-204, XP000960515 ISSN: 0340-3696
- D3: LIMAT ALAIN ET AL: "Successful treatment of chronic leg ulcers with epidermal equivalents generated from cultured autologous outer root sheath cells." JOURNAL OF INVESTIGATIVE DERMATOLOGY, vol. 107, no. 1, 1996, pages 128-135, XP000960501 ISSN: 0022-202X

2. The present application discloses that skin equivalents can be obtained in organotypic cultures from outer root sheath (ORS) cells derived from culturing anagen or growing hair *in toto*. The culture conditions include (i) culturing of the hair *in toto* upon microporous membranes carrying human fibroblast feeder cells at their under-surface, (ii) seeding of the thus obtained ORS cells upon a modified microporous membrane carrying feeder cells at their under-surface, where they differentiate to form an epidermal equivalent, (iii) using a reduced concentration of allogenic or homologous serum and substances isolated from blood components, (iv) devising a method to transport the epidermal equivalents that reduces the probability of mechanical damage.

3. **Novelty** (Article 33(2) PCT)

Each of the independent claims is directed to methods relating to the culture of intact hair follicles of an anagenic hair to obtain outer root sheath cells. The IPEA notes that this feature is not disclosed in the prior art and thus claims 1-46 are novel under the terms of Article 33(2) PCT.



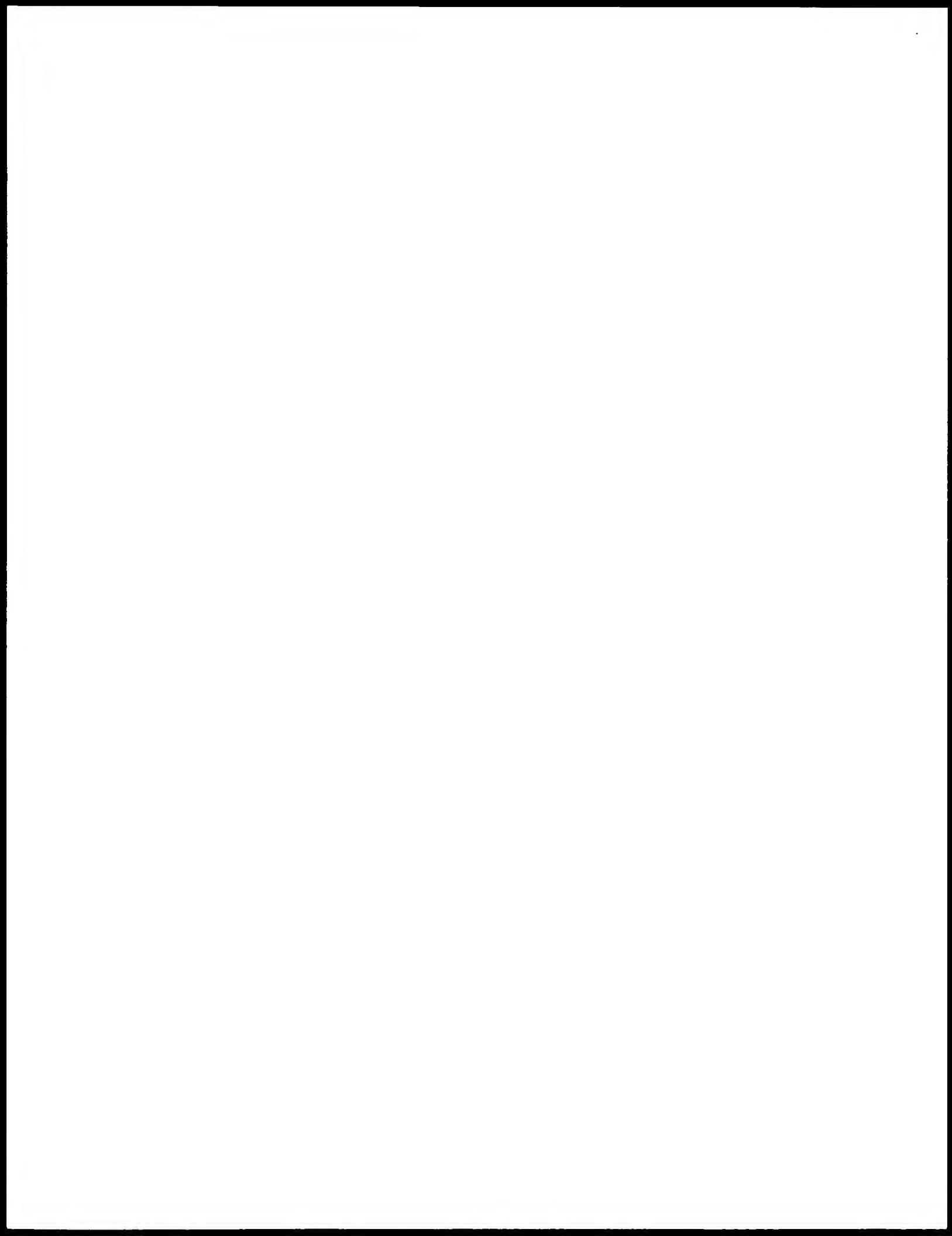
**4. Inventive step (Article 33(3) PCT)**

**D1** is a patent application by the same inventors on the use of 10-15% (functioning range 3-60%) autologous or homologous serum in the culture of dermal equivalents from ORS cells (column 3, line 24). A further increase in cell growth was achieved by the addition of 0.05-1% autologous or homologous platelet released peptides (=releasates) (column 3, lines 56-65). The dermal equivalents were seeded on hyaluronic acid membranes or other biodegradable material prior to transplantation to optimise handling (column 4, lines 26-36).

**D2** discloses the culture of ORS cells from plucked anagen hair *in toto*, without prior removal of the bulbs (i.e. intact hair follicles). Specifically, lines 6-7 of column 1 on page 198 states that "in some experiments, the bulbs were removed using scissors", implicating that in others, the bulbs were not removed and the intact hair follicle was cultured. ORS cells are grown on dead de-epidermised dermis as opposed to human dermal fibroblasts. **D3** is a publication by the inventors on basically the same technique as the application except (i) the culturing of hair *in toto*, (ii) the culturing in <5% human serum and (iii) the coating with fibrin glue. At the relevant filing date of the present application, the skilled person in search for optimised treatments of patients in need of skin grafts would have combined the teaching on *in toto* hair culture of **D2**, with the teaching on the use of human serum and releasates of **D1** and the general teachings of **D3**, to arrive at the subject-matter of present claims 1-7, 9-12, and 28-38. Said claims are thus found to lack an inventive step under the terms of Article 33(3) PCT.

**5. Industrial applicability (Article 33(4) PCT)**

The subject-matter of claims on which an opinion can be formed (see item III) appears to be industrially applicable under the terms of Article 33(4) PCT.



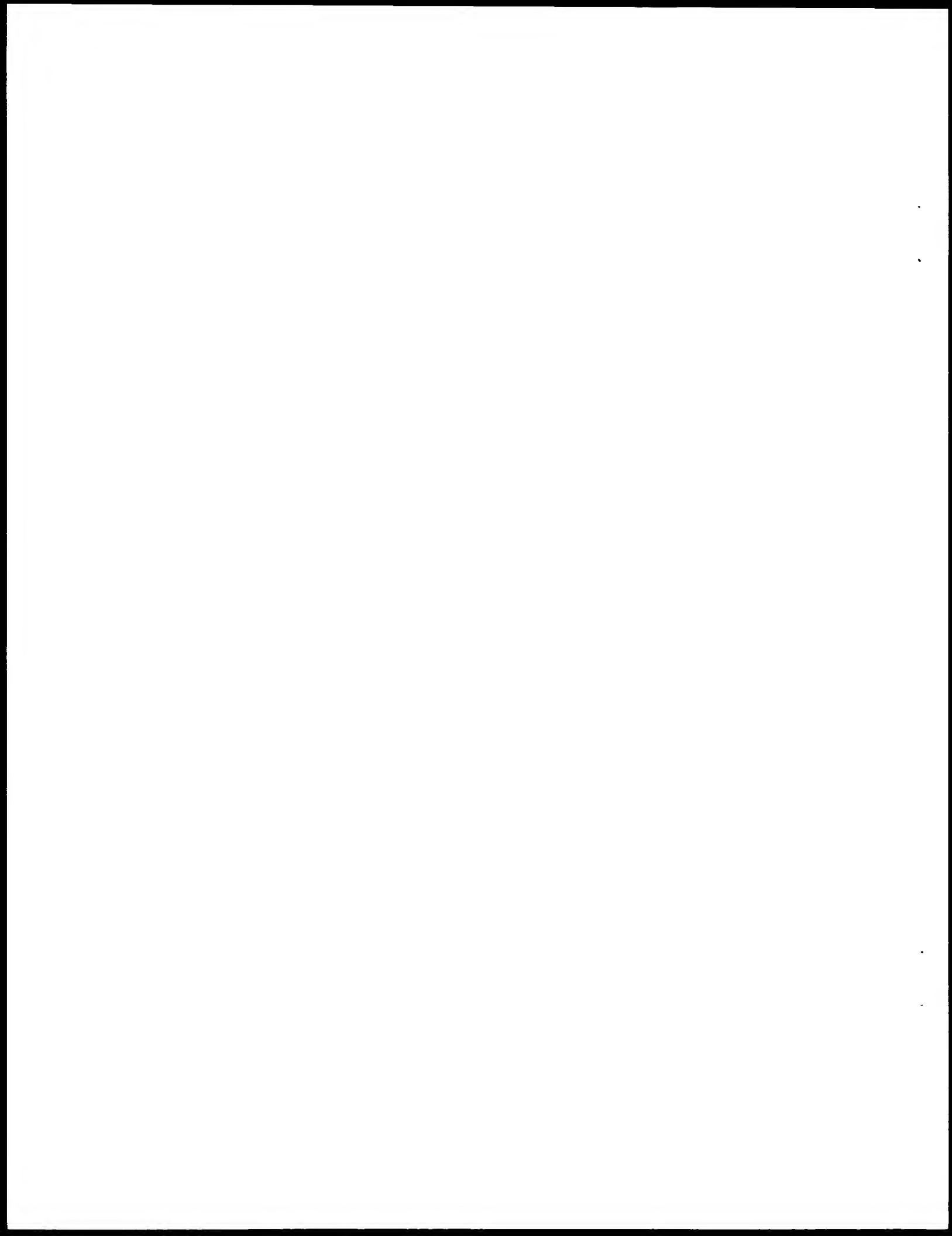
few, noncornified cell layers (Hetton *et al.*, 14 J. Am. Acad. Dermatol. 399-405 (1986); Leigh & Purkis, 11 Clin. Exp. Dermatol. 650-652 (1986); Leigh *et al.*, 117 Brit. J. Dermatol. 591-597 (1987); Harris *et al.*, 18 Clin. Exp. Dermatol. 417-420 (1993)), trypsinized single cells attached to collagen-coated dressings (Brysk *et al.*, 25 J. Am. Acad. Dermatol. 238-244 (1991)), skin equivalents (Mol *et al.*, 24 J. Am. Acad. Dermatol. 77-82 (1991)) has yet to be convincingly documented within the scientific literature. The same lack of quantitative findings also holds true for various reports on the grafting of freshly isolated, autologous interfollicular keratinocytes (Hunyadi *et al.*, 14 J. Dermatol. Surg. Oncol. 75-78 (1988)) or ORS cells (Moll *et al.*, 10 46 Hautarzt 548-552 (1995)) fixed to the wound bed by the use of a fibrin glue. However, it should be noted that the disadvantages of the bovine serum used during cultivation of the keratinocytes may contribute to reduced "take" rate, due to the fact that it resists in keratinocytes (*see e.g.*, Johnson *et al.*, 11 J. Burn Care Rehab. 504-509 (1990)).

15

#### SUMMARY OF THE INVENTION

Prior to the disclosure of the present invention herein, the standard methodology for the generation of a primary culture of ORS keratinocytes consisted of the plucking of an anagen (*i.e.*, growing hair shaft) hair followed by a careful 20 microscopic dissection to remove the hair bulbs and the infundibular hair shaft. The resulting outer root sheath was then placed on the culture insert for initiation of the primary keratinocyte culture. However, numerous subsequent studies (approximately 200), wherein the anagen hair was placed directly on the culture insert without performing the initial micro-dissection to remove the hair bulbs and the infundibular 25 hair shaft, have demonstrated that such tedious and time-consuming dissection of the plucked anagen hair was not required. This has served to markedly simplify the handling process, reduce the risk for contamination, and resulted in more efficient initiation of keratinocyte cell plating.

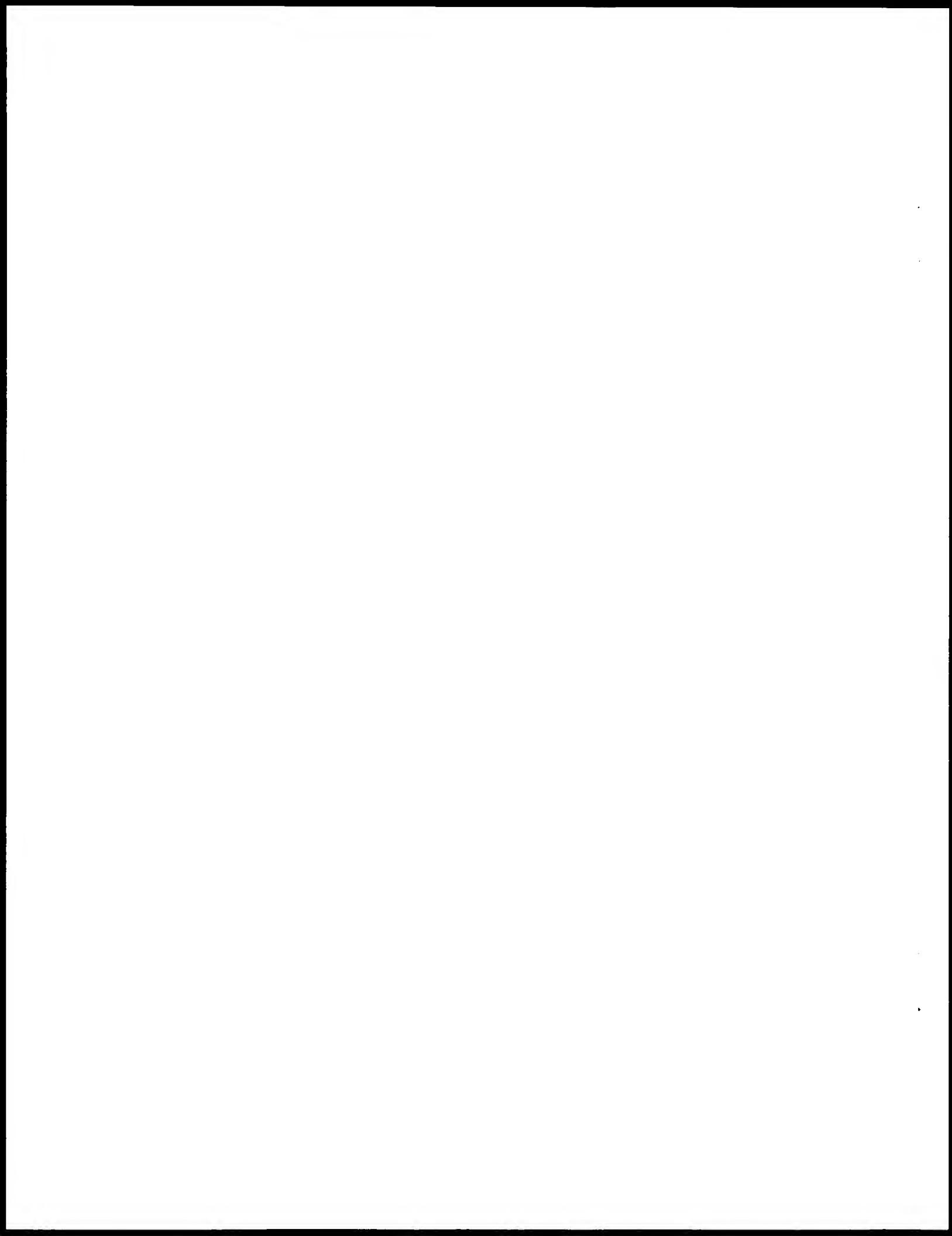
Accordingly, it is an object of the present invention to provide improved and 30 simplified methods for the generation of keratinocytes or keratinocyte precursors from outer root sheath cells (ORS cells) in fully defined culture conditions for the treatment of various types of skin defects (*e.g.*, chronic wounds such as leg ulcers, diabetic



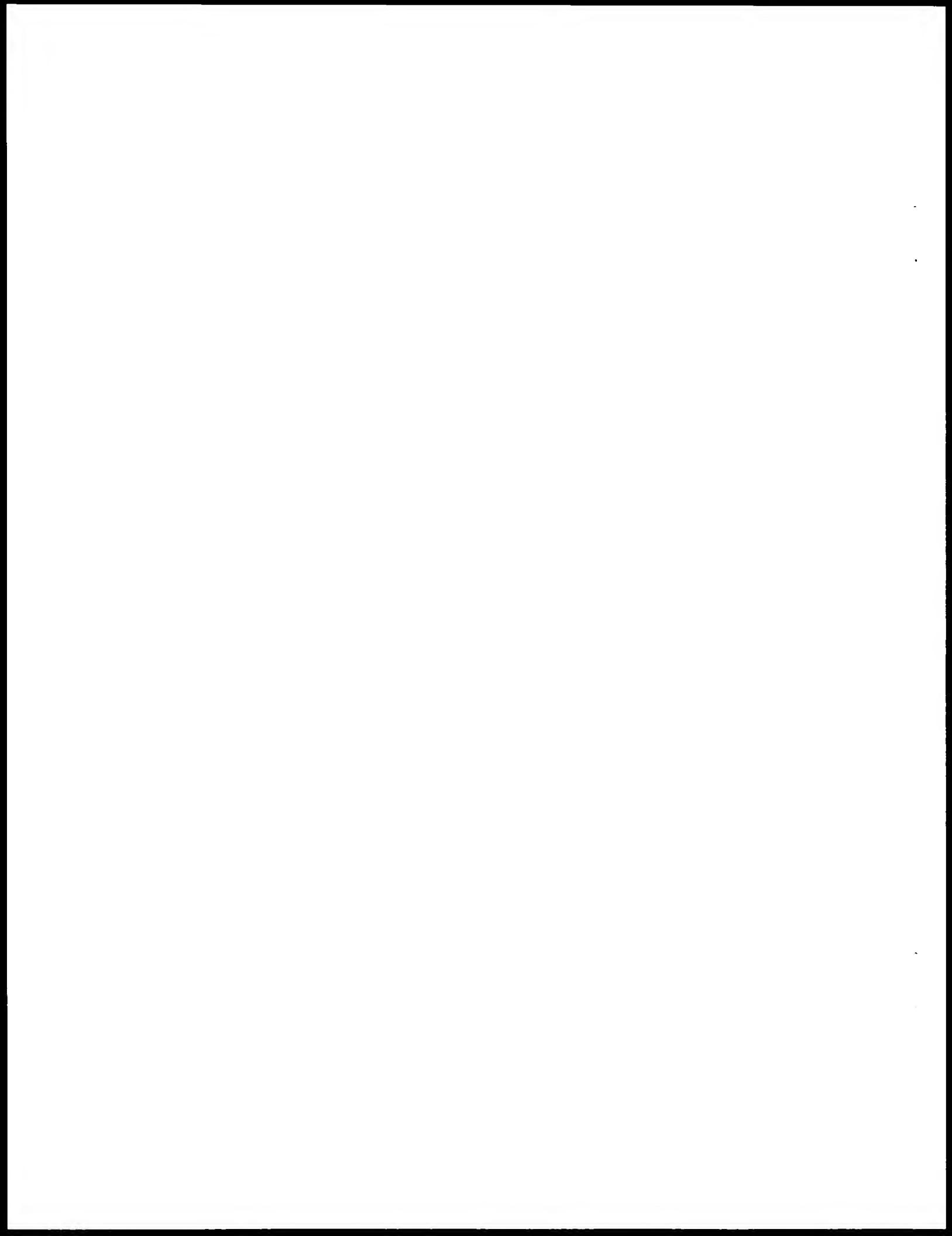
## CLAIMS

## WE CLAIM:

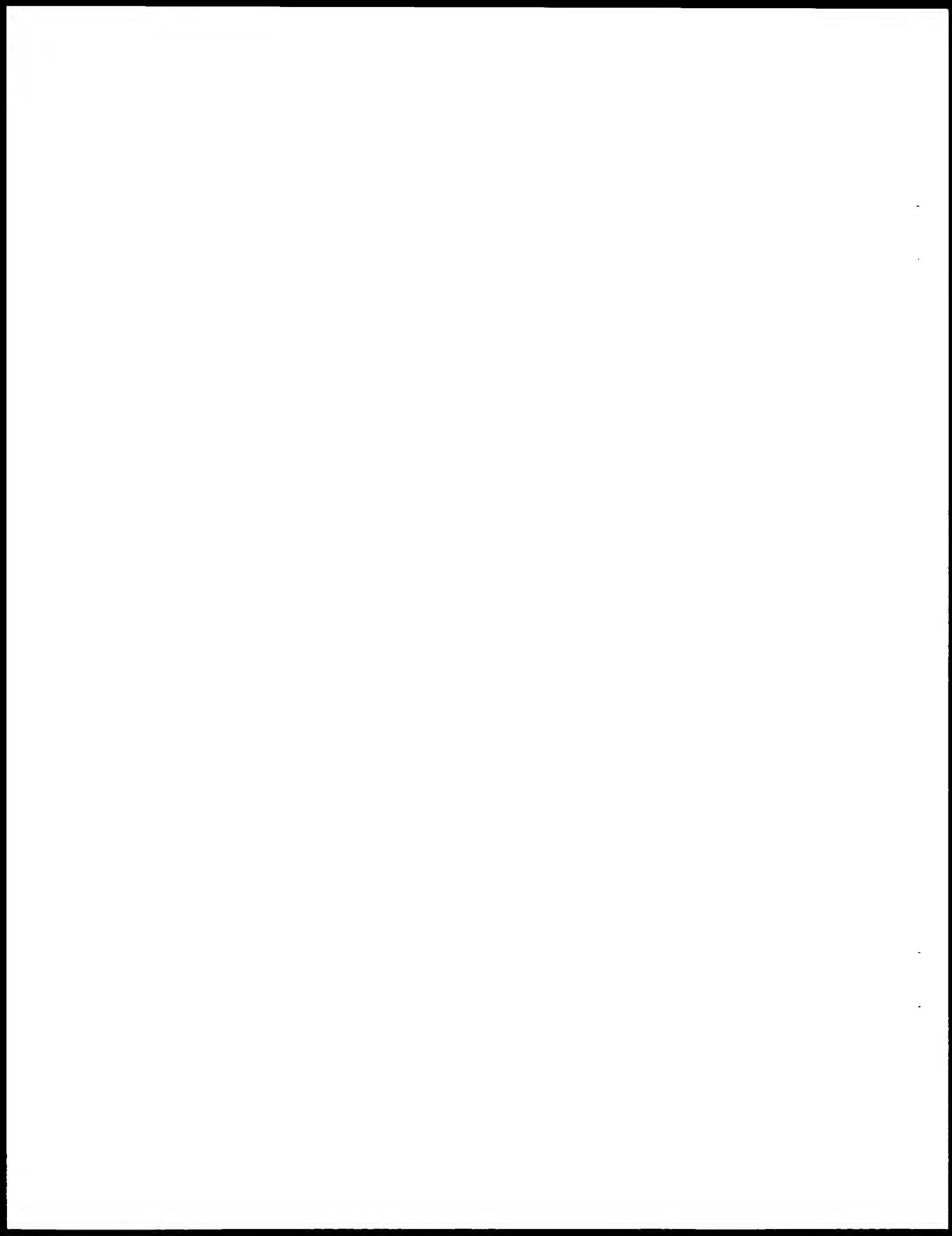
- 5        1. A method for the treatment of a skin defect comprising the application, to said defect, of a portion of an epidermal or complex skin equivalent comprising keratinocyte precursor cells derived from the culturing of outer root sheath cells which were initially derived from the culturing, *in toto*, of an anagen or growing hair.
- 10        2. The method of claim 1, wherein said outer root sheath cells are autologous cells derived from the individual who will subsequently undergo treatment for a skin defect.
- 15        3. The method of claim 1, wherein said outer root sheath cells are homologous cells.
- 20        4. The method of claim 1, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing only homologous or autologous biological supplements.
- 25        5. The method of claim 1, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing human serum in a concentration of less than 5%.
- 30        6. The method of claim 1, wherein the culture density of said keratinocyte precursor cells is between about  $3 \times 10^4$  cells/cm<sup>2</sup> and about  $1 \times 10^5$  cells/cm<sup>2</sup>.
7. The method of claim 1, wherein said epidermal or skin equivalents are coated on their top or cornified side with a fibrin glue.
8. The method of claim 1, wherein said epidermal or skin equivalents are coated on their top or cornified side with a carrier membrane.



9. The method of claim 1, wherein the graft thickness for said epidermal equivalents is 50-150 microns.
- 5 10. The method of claim 1, wherein the graft thickness for said complex skin equivalents does not exceed 0.4 mm.
- 10 11. A method for the treatment of a skin defect comprising the application, to said defect, of a portion of an epidermal or skin equivalent comprising keratinocyte precursor cells derived from the culturing of outer root sheath cells which were initially derived from the culturing of an anagen or growing hair; wherein all culturing of cells is performed in a medium which utilizes autologous or homologous human serum in a concentration of less than about 5%.
- 15 12. The method of claim 11, wherein said anagen or growing hair is cultured *in toto*.
- 20 13. The method of claim 11, wherein said outer root sheath cells are autologous cells derived from the individual who will subsequently undergo treatment for a skin defect.
14. The method of claim 11, wherein said outer root sheath cells are homologous cells.
- 25 15. The method of claim 11, wherein the culture density of said keratinocyte precursor cells is between about  $3 \times 10^4$  cells/cm<sup>2</sup> and about  $1 \times 10^5$  cells/cm<sup>2</sup>.
- 30 16. The method of claim 11, wherein said epidermal or skin equivalents are coated on their top or cornified side with a fibrin glue.



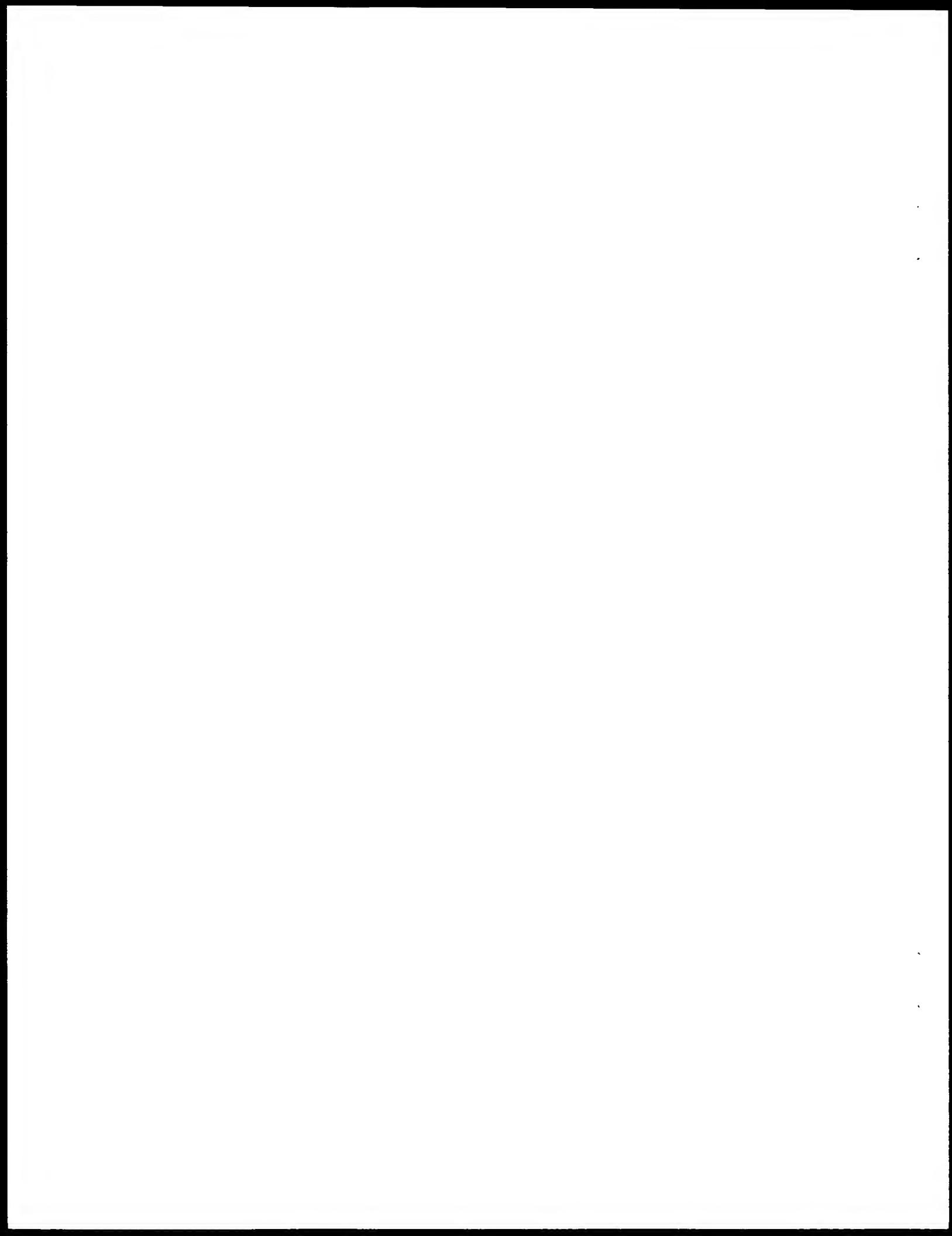
17. The method of claim 11, wherein said epidermal or skin equivalents are coated on their top or cornified side with a carrier membrane.
18. The method of claim 11, wherein the graft thickness for said epidermal equivalents is 50-150 microns.  
5
19. The method of claim 11, wherein the graft thickness for said complex skin equivalents does not exceed 0.4 mm.  
10
20. A method for the treatment of a skin defect comprising the application, to said defect, of a portion of an epidermal or skin equivalent comprising keratinocyte precursor cells derived from the culturing of outer root sheath cells which were initially derived from the culturing of an anagen or growing hair; wherein said epidermal or skin equivalent are coated on their top or cornified side with a fibrin glue.  
15
21. The method of claim 20, wherein said anagen or growing hair is cultured *in toto*.  
20
22. The method of claim 20, wherein said outer root sheath cells are autologous cells derived from the individual who will subsequently undergo treatment for a skin defect.  
25
23. The method of claim 20, wherein said outer root sheath cells are homologous cells.  
24. The method of claim 20, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing only homologous or autologous biological supplements.  
30



25. The method of claim 20, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing human serum in a concentration of less than 5%.
- 5 26. The method of claim 20, wherein the culture density of said keratinocyte precursor cells is between about  $3 \times 10^4$  cells/cm<sup>2</sup> and about  $1 \times 10^5$  cells/cm<sup>2</sup>.
- 10 27. The method of claim 20, wherein said epidermal or skin equivalents are coated on their top or cornified side with a fibrin glue which contains one or more anti-microbial, anti-fungal, or anti-viral agents emulsified therein.
28. A method for the selection of keratinocyte precursor cells from the outer root sheath of hair for subsequent use in a composition for healing a skin defect, comprising the steps of:
  - 15 (a) plucking of an anagen or growing hair;
  - (b) primary-culturing the outer root sheath-derived keratinocyte precursor cells by adhering said anagen hair, *in toto*, to a microporous membrane, which possesses growth-arrested/limited feeder cells on its undersurface so as to select for keratinocyte precursor cells from the outer root sheath of hair;
  - 20 (c) organotypically-culturing the outer root sheath cells harvested from said primary cultures by inoculating a microporous membrane which also possesses growth-arrested/limited feeder cells on its undersurface;
  - (d) generating an epidermal or complex skin equivalent, for subsequent use as a graft insert, comprised of keratinocyte precursor cells by placing a carrier membrane on top of said organotypic-culture from step (c) and detaching said skin or epidermal equivalent, which is comprised of the keratinocyte precursor cells and carrier membrane, together as a single, laminar unit;
  - 25 (e) contacting said epidermal or skin equivalent with a skin defect present on an individual, and immobilizing said epidermal or skin equivalent at the site of contact.



29. The method of claim 28, wherein said outer root sheath cells are autologous cells derived from the individual who will subsequently undergo treatment for a skin defect.  
5
30. The method of claim 28, wherein said outer root sheath cells are homologous cells.
31. The method of claim 28, wherein the culture density of said keratinocyte precursor cells is between about  $3 \times 10^4$  cells/cm<sup>2</sup> and about  $1 \times 10^5$  cells/cm<sup>2</sup>.  
10
32. The method of claim 28, wherein the culture density of said growth-arrested/limited feeder cells on said microporous membrane is between about  $1 \times 10^4$  cells/cm<sup>2</sup> and about  $5 \times 10^4$  cells/cm<sup>2</sup>.  
15
33. The method of claim 28, wherein said growth-arrested/limited feeder cells are banked or immortalized cells.
34. The method of claim 28, wherein said primary and organotypic cultures utilize autologous or homologous human serum.  
20
35. The method of claims 28, wherein said primary and organotypic cultures utilize autologous or homologous human serum in a concentration of less than about 5%.  
25
36. The method of claim 28, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing only homologous or autologous biological supplements.
37. The method of claim 28, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing only homologous or autologous releasates from blood components.  
30



38. The method of claims 28, wherein said epidermal or skin equivalent comprises outer root sheath cells cultured in a medium containing only homologous or autologous releasates from blood components at a concentration of about 0.1% to about 20%.  
5
39. The method of claim 28, wherein said epidermal equivalents are coated on their top or cornified side with a fibrin glue.  
10
40. The method of claims 28, wherein said epidermal equivalents are coated on their top or cornified side with a carrier membrane.  
15
41. The method of claims 28, wherein the graft thickness for said epidermal equivalents is 50-150 microns.  
20
42. The method of claims 28, wherein the graft thickness for said complex skin equivalents does not exceed 0.4 mm.  
25
43. The method of claim 28, wherein said microporous membrane is coated by one or ore extracellular matrix substances selected from a group consisting of: fibrin, fibronectin, collagens, laminins and hyaluronan.  
30
44. The method of claims 28, wherein said microporous membrane possesses a growth-arrested/limited feeder cell system on its undersurface with said feeder cells of at least one type of cell selected from the group comprising human dermal fibroblasts, epidermal cells, mesenchymal cells, neuronal cells and endothelial cells.  
35
45. The method of claim 28, wherein said carrier membrane is made from one or more types of materials selected from the group comprising polyester, PTFE, polyurethane, hyaluronic acid, polylactic acid, collagen, or a silicone or vaseline gauze dressing.



46. The method of claim 28, wherein the size of said epidermal equivalent is selected from the group consisting of 1.0 cm, 1.5 cm, 2.0 cm, and 2.5 cm in diameter.

5

47. A method of shipping or transporting epidermal equivalents comprising:

- (a) detaching said epidermal equivalents from a culture medium, and
- (b) transferring said epidermal equivalents onto a transport medium.

10 48. The method of claim 47, wherein said epidermal equivalents are coated on their top or cornified side with a carrier membrane.

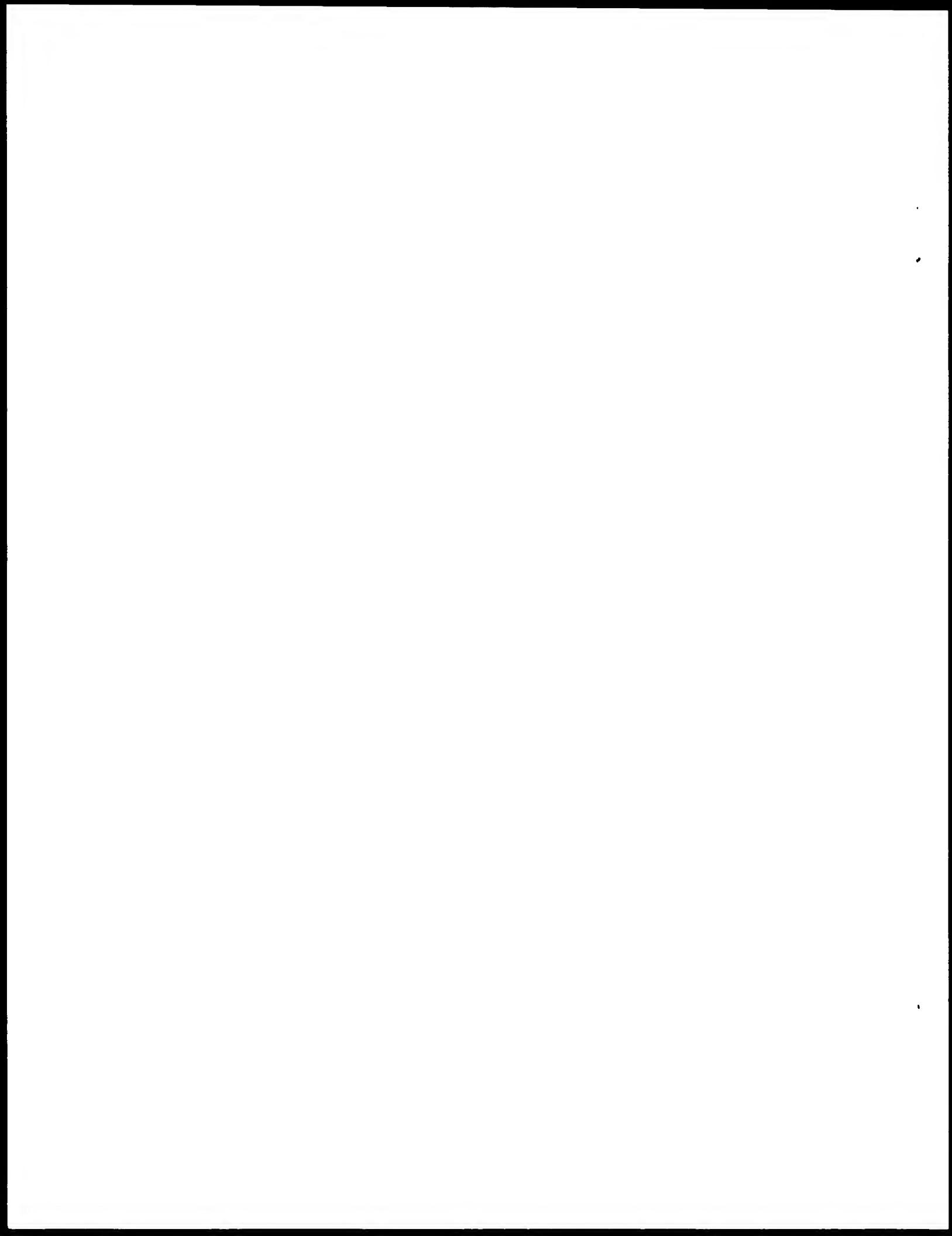
49. The method of claim 48, wherein said epidermal equivalents are further sealed and shipped for future use in grafting.

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50. The method of claim 47, wherein said transport medium comprises a solidified or gelled medium.

20

51. The method of claim 50, wherein said solidified or gelled medium is selected from the group consisting of agarose, methyl cellulose, or another gelifying substance.



**PATENT COOPERATION TREATY**  
**PCT**

**INTERNATIONAL SEARCH REPORT**

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference  GWS/MRM/22559	<b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No.  PCT/IB 00/01076	International filing date (day/month/year)  20/07/2000	(Earliest) Priority Date (day/month/year)  20/07/1999
Applicant  EPITECH S.A.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

It is also accompanied by a copy of each prior art document cited in this report.

**1. Basis of the report**

- a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
  - the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).
- b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :
  - contained in the international application in written form.
  - filed together with the international application in computer readable form.
  - furnished subsequently to this Authority in written form.
  - furnished subsequently to this Authority in computer readable form.
  - the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
  - the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished
- 2.  **Certain claims were found unsearchable** (See Box I).
- 3.  **Unity of invention is lacking** (see Box II).
- 4. With regard to the **title**,
  - the text is approved as submitted by the applicant.
  - the text has been established by this Authority to read as follows:

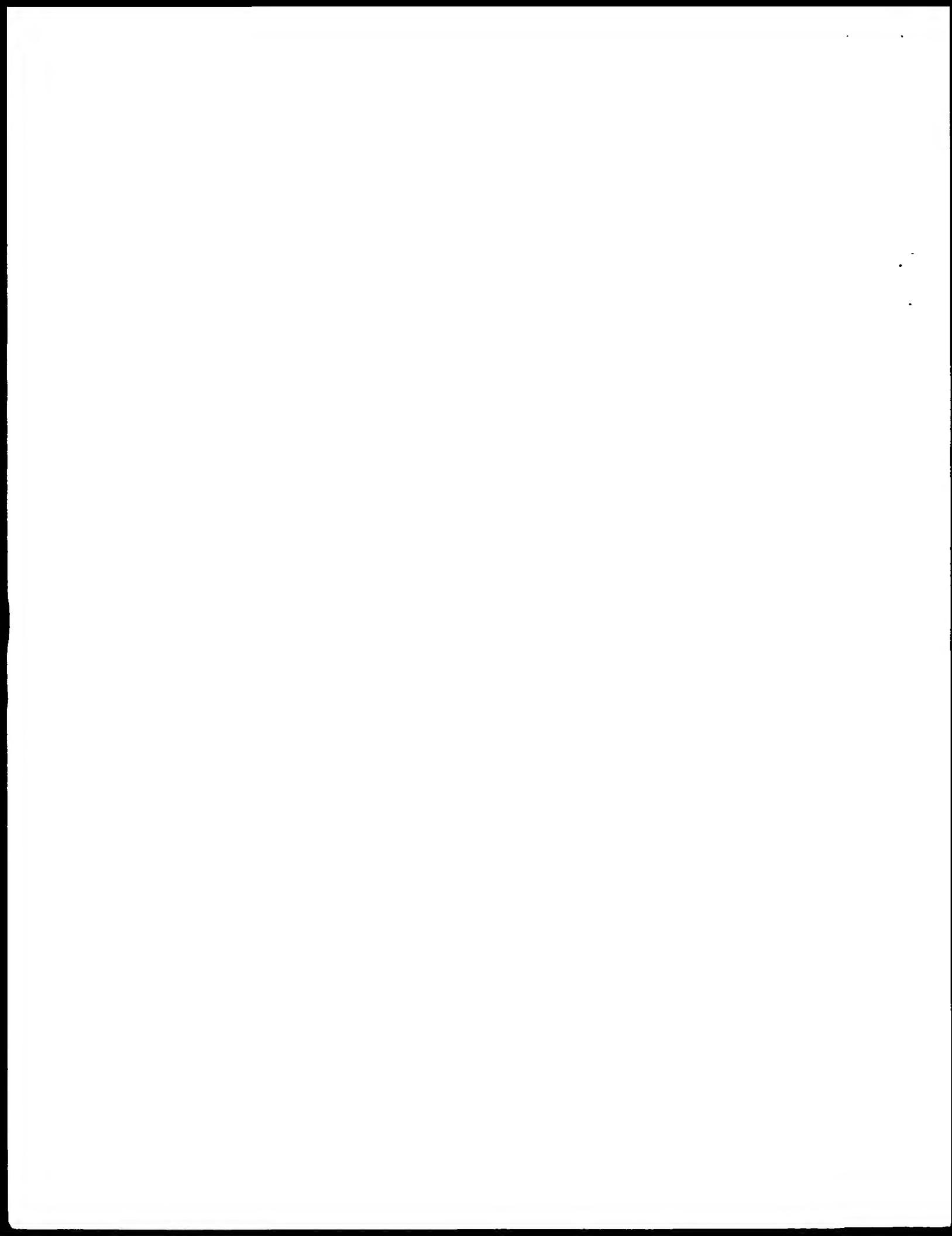
5. With regard to the **abstract**,

- the text is approved as submitted by the applicant.
- the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

- as suggested by the applicant.
- because the applicant failed to suggest a figure.
- because this figure better characterizes the invention.

None of the figures.



**INTERNAL SEARCH REPORT**International application No.  
PCT/IB 00/01076**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:  
**Although claims 1–46 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.**
2.  Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

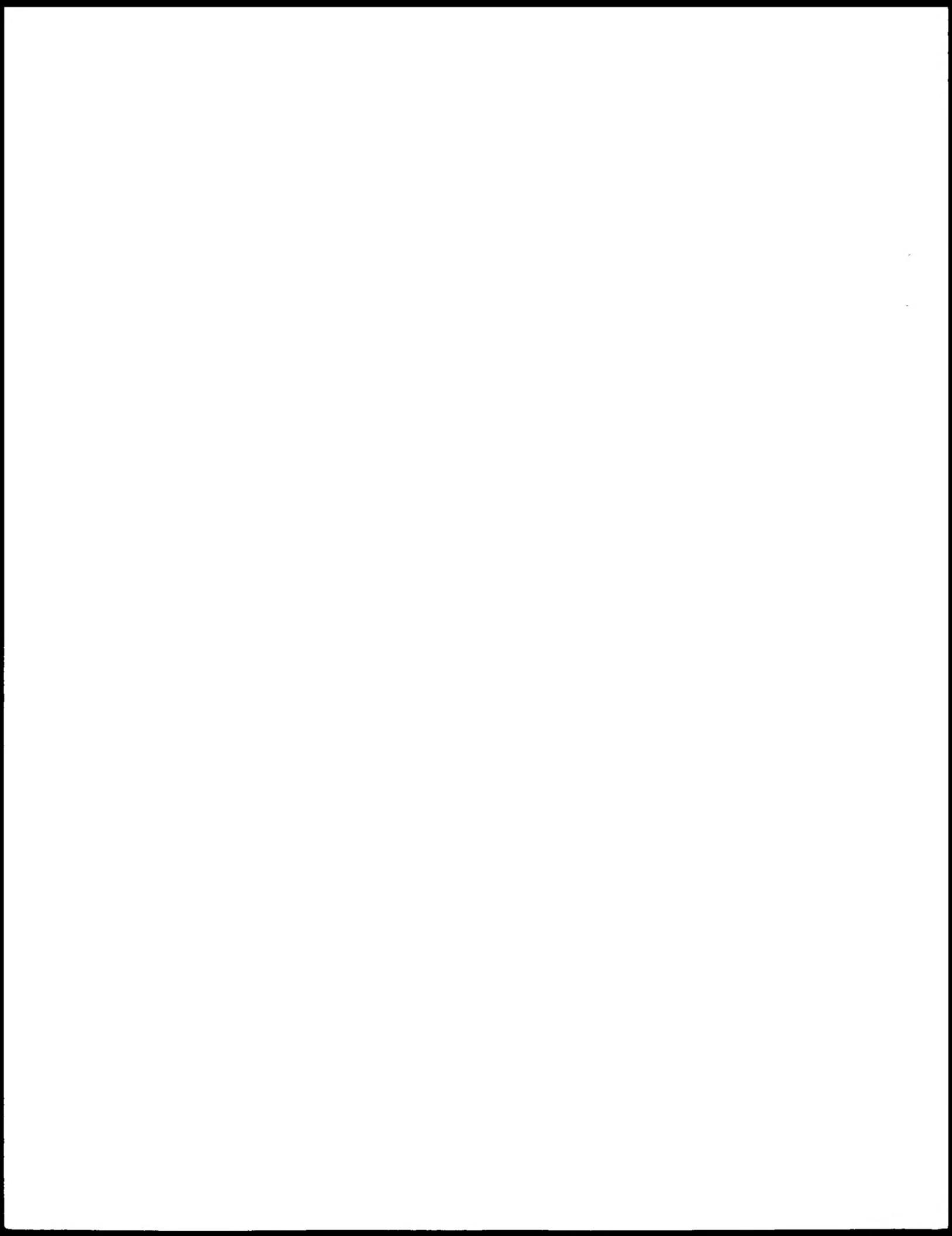
This International Searching Authority found multiple inventions in this international application, as follows:

**see additional sheet**

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

- The additional search fees were accompanied by the applicant's protest.
- No protest accompanied the payment of additional search fees.



FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-46

1.1. Claims: 1-3, 6, 9, 10 all completely and 4, 5, 7, 8, 12, 21, 28-46 all partially

A method for the generation of an epidermal equivalent from keratinocyte precursors from outer root sheath (ORS) cells by culture of anagen hair in toto. A method for treating skin defects by applying a skin equivalent thus obtained.

1.2. Claims: 11, 13-15, 18, 19 all completely and 4, 5, 12, 16, 17, 24, 25, 34-38 all partially

A method for the generation of an epidermal equivalent from keratinocyte precursors from outer root sheath (ORS) cells by culturing in media supplemented with less than 5% autologous or homologous human serum . A method for treating skin defects by applying a skin equivalent thus obtained.

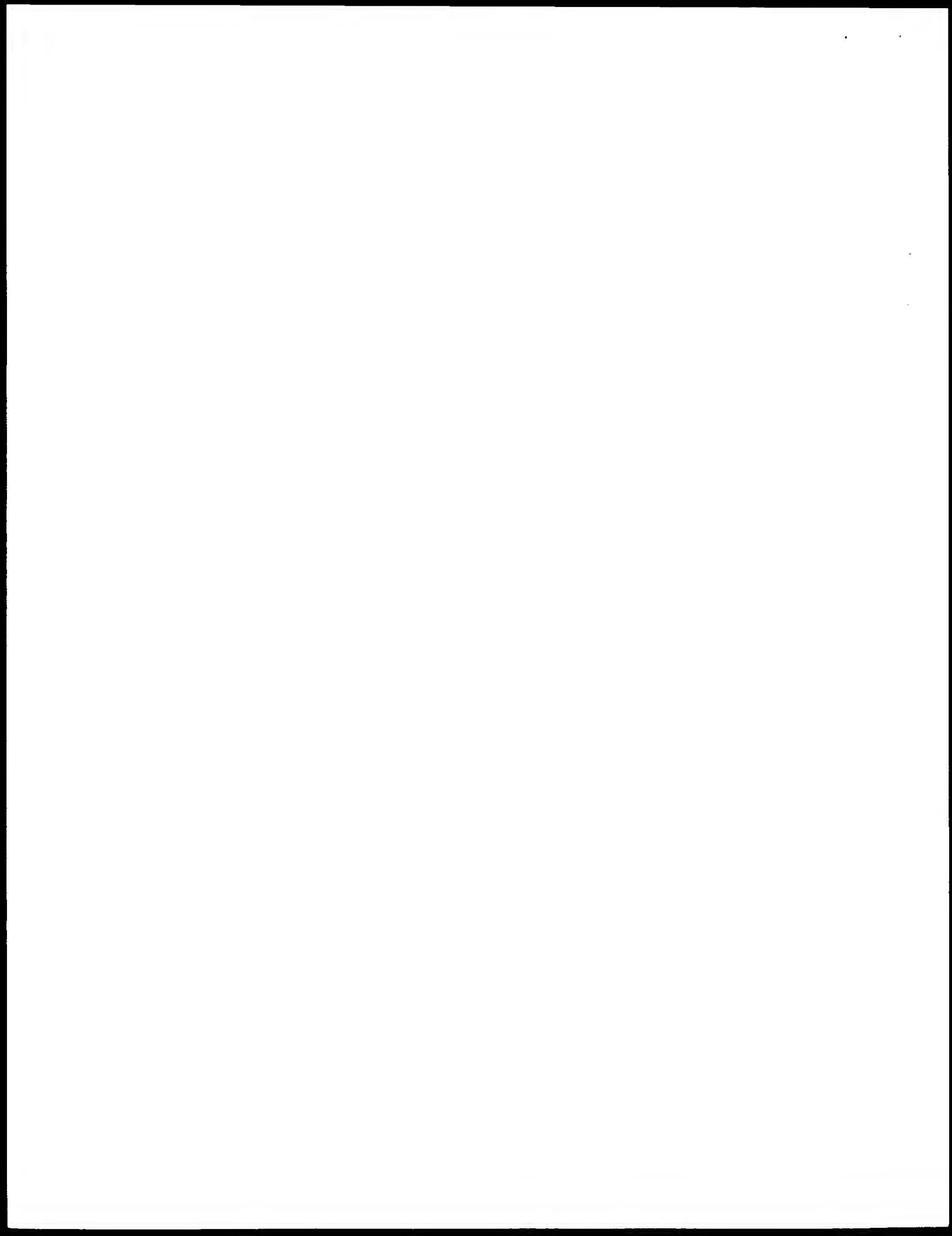
1.3. Claims: 20, 22, 23, 26, 27 all completely and 7, 8, 16, 17, 21, 24, 25, 28-46 all partially

A method for the generation of an eidermal equivalent from keratinocyte precursors from outer root sheath (ORS) cells wherein said epidermal equivalent is coated on its top side with a fibrin glue. A method for treating skin defects by applying a skin equivalent thus obtained.

2. Claims: 47-51

A method of shipping or transporting epidermal equivalents

Please note that all inventions mentioned under item 1, although not necessarily linked by a common inventive concept, could be searched without effort justifying an additional fee.



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/IB 00/01076

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N5/06 A61K35/36 A61P17/02

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 C12N A61K A61P C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, MEDLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE 196 51 992 A (TOLOCZYKI CHRISTIAN DR) 25 June 1998 (1998-06-25)	11, 13-15, 17-19, 46 4, 5, 12, 18, 19, 34-38
Y	column 3, line 23 -column 4, line 59 --- LENOIR-VIALE M -C ET AL: "Epidermis reconstructed from the outer root sheath of human hair follicle: Effect of retinoic acid." ARCHIVES OF DERMATOLOGICAL RESEARCH, vol. 285, no. 4, 1993, pages 197-204, XP000960515 - ISSN: 0340-3696 page 198, left-hand column, line 5 - line 7 --- -/-	1-7, 9, 10, 12, 18, 19, 28-38

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

° Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*&\* document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

7 February 2001

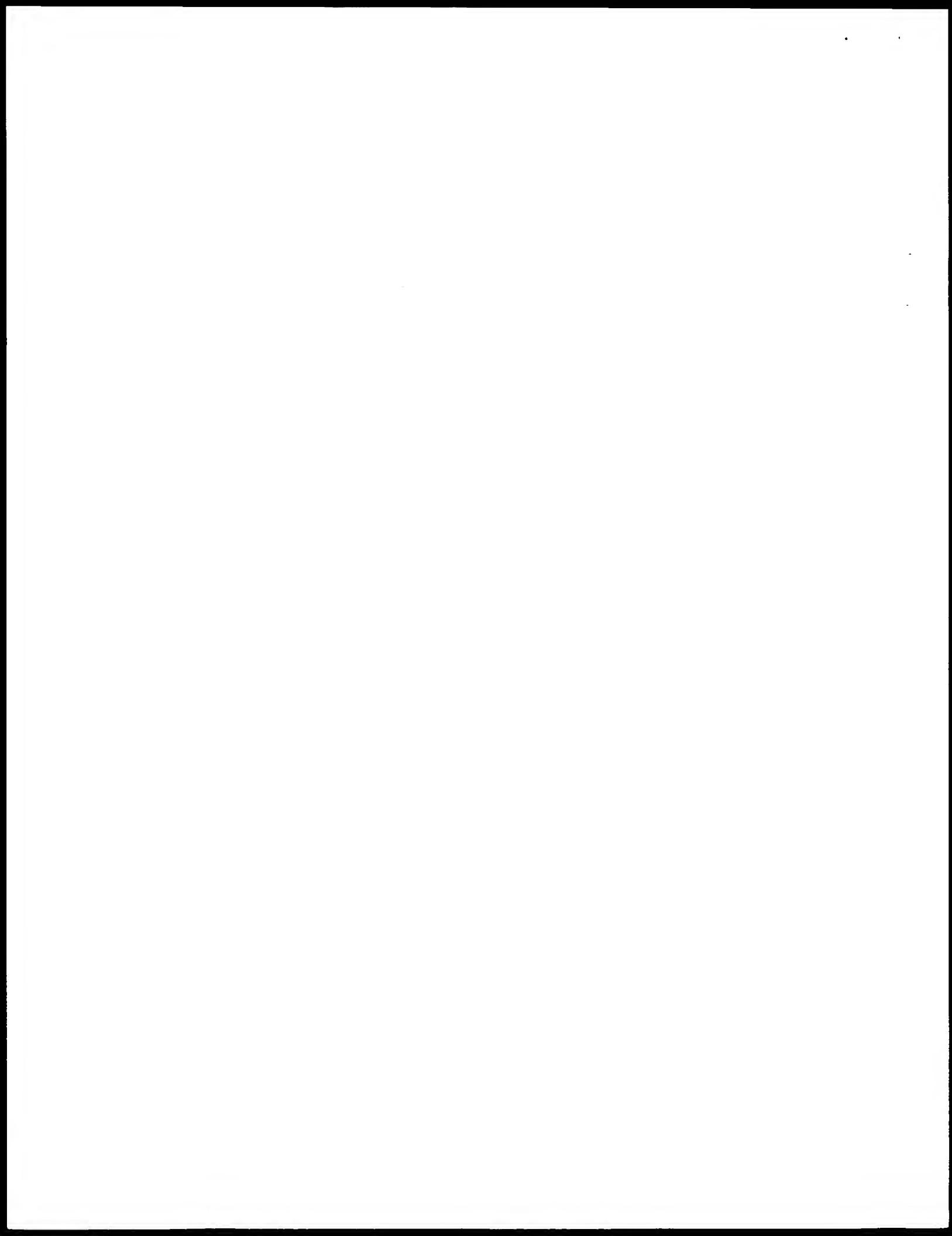
22.02.01

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Nichogianopoulou, A



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/IB 00/01076

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LIMAT ALAIN ET AL: "Successful treatment of chronic leg ulcers with epidermal equivalents generated from cultured autologous outer root sheath cells." JOURNAL OF INVESTIGATIVE DERMATOLOGY, vol. 107, no. 1, 1996, pages 128-135, XP000960501 ISSN: 0022-202X the whole document ---	1-7, 9, 10, 12, 28-38
P, X	US 5 968 546 A (BAUR MARCUS ET AL) 19 October 1999 (1999-10-19) the whole document ---	1-46
X	WO 93 08776 A (CULTURE TECHNOLOGY INC) 13 May 1993 (1993-05-13) the whole document -----	47-51



**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International Application No

PCT/IB 00/01076

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE 19651992 A	25-06-1998	NONE	
US 5968546 A	19-10-1999	NONE	
WO 9308776 A	13-05-1993	NONE	

